REFLEX AND NEUROENDOCRINE MECHANISMS OF CATECHOLAMINE RELEASE FROM THE ADRENAL MEDULLA

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SUMMARY

Two distinct mechanisms of catecholamine release from the adrenal medulla were investigated.

One was whether the two catecholamines, noradrenaline and adrenaline, could be released selectively by different reflex or pharmacological stimuli to the adrenal gland.

The other was whether a humoral mechanism involving the anterior pituitary - adrenocortical axis participated in the regulation of adrenal catecholamine secretion.

We have studied these phenomena in anaesthetised cats and dogs in which we independently elicited the baroreceptor and chemoreceptor reflexes, and in isolated Locke - perfused adrenal glands subjected to pharmacological stimulation by specific muscarinic and nicotinic agonists.

Techniques were established:-

a) For constant pressure perfusion of the carotid bifurcation using a servo control amplifier system.

b) For chemoreceptor stimulation by using the reducing agent, sodium dithionite, to lower the oxygen tension of the blood.

c) Isolated adrenal gland perfusion with automated sample collection and differential catecholamine assay.

In Dogs no evidence for selective release was found.

1) The incremental releases mediated by the baroreceptor and chemoreceptor reflexes and by splanchnic nerve stimulation contained the same proportion of noradrenaline to adrenaline (i.e. 1:4) which was equal to that found in the resting output.

2) This proportion of noradrenaline to adrenaline was also found in the increments released in response to specific nicotinic and muscarinic stimulation using hppTMA and acetyl-β-Methyl Choline respectively.

In Cats we did find evidence of selective catecholamine release with both reflex and pharmacological stimulation.

1) Baroreceptor stimulation released an increment containing predominantly noradrenaline (75% noradrenaline).

2) Chemoreceptor stimulation released an increment containing predominantly adrenaline (16% noradrenaline)

3) Both of the mean percentages were significantly different ($P < 0.01$) from one another and from that in the resting output which contained 46% noradrenaline.

4) Nicotinic (hppTMA) stimulation released an increment which contained significantly ($P < 0.01$) more noradrenaline (57% noradrenaline) than that released by muscarinic (methacholine) stimulation (34% noradrenaline). However, at none of the doses tested was there any evidence that either drug released solely one catecholamine.

These results suggest that with respect to the above reflexes and drugs, there is a species difference between the dog and the cat regarding the selective release of adrenal catecholamines.

Our evidence for the participation of a humoral component, via the anterior pituitary - adrenocortical axis, in the control of adrenal catecholamine output in the dog is as follows:-

1) We found that the release of catecholamines provoked by chemoreceptor stimulation outlasts the stimulus and usually continues to rise after its cessation.

2) This delayed component of the adrenal catecholamine discharge in response to chemoreceptor stimulation was seen in a denervated gland. Denervation abolished the immediate catecholamine release in response to chemoreceptor stimulation and the baroreceptor reflex.

3) Chemoreceptor stimulation has been shown by other workers to release corticotrophin from the pituitary gland.

4) We found that intravenous Synacthen releases catecholamines from both intact and denervated adrenal glands.

5) The delayed release of catecholamines in response to both Synacthen and chemoreceptor stimulation is inhibited by cycloheximide, which has been shown by other workers to block steroid secretion. There was no evidence that cycloheximide had any effect on neuronally mediated adrenal medullary release.

6) Hydrocortisone, in the order of concentration found in adrenal venous blood after stress, was shown to increase the catecholamine output from the isolated Locke perfused adrenal gland in a dose dependent manner.

I have included in the appendices some of the other work which we did concurrently with the above studies.

We present evidence, from experiments done in man, that increased concentrations of arterial plasma noradrenaline contribute to the hypoxic potentiation of the respiratory response to moderate exercise. Techniques are discussed for the estimation of catecholamines and their ortho-methylated metabolites in peripheral plasma and urine.

Our technique for measuring venous capacitance in the lower half of the body is described and evidence is presented which suggests that there is an interaction between portal and systemic capacitance which could have unexpected effects on venous return.

Part of our work has been published previously

-see list at end of thesis.

CONTENTS

Outline of Research

We investigated two separate aspects of catecholamine release from the adrenal medulla.

Firstly, whether the two catecholamines, noradrenaline and adrenaline, could be released independently by different reflex stimuli to the gland.

Secondly, whether a humoral mechanism involving the anterior pituitary-adrenocortical axis participated in the regulation of catecholamine secretion.

Most authorities now accept that noradrenaline and adrenaline are stored within the adrenal medulla in clusters of cells containing one or other catecholamine but there is controversy over whether the two catecholamines are under independent secretory control.

We describe this phenomenon as selective release and define it as the ability of different reflex stimuli to release from the adrenal medulla, mixtures of catecholamines containing significantly different ratios of noradrenaline to adrenaline in the increment secreted in excess of the resting output. We distinguish selective release from preferential release. A reflex may preferentially release more adrenaline than noradrenaline because this is the proportion in which the two catecholamines are stored in a releasable form within the gland. Selective release is only demonstrated when two distinct reflexes result in incremental releases of equivalent magnitude which contain significantly different proportions of the two hormones.

Since 1949, differential assays have been used to measure the noradrenaline and adrenaline secreted in response to various reflex stimuli. Some workers are adamant that selective release does not occur while others present evidence in favour of the independent release of noradrenaline and adrenaline by a number of different stimuli. The majority of these experiments have been done using dogs or cats and on detailed examination of the literature, we found convincing evidence of selective release only in those experiments done on cats. Those workers who used dogs either did not find selective release or their results did not justify their conclusion.

To our knowledge, the responses to the same pair of reflex stimuli had not been compared satisfactorily by using similar techniques in both dogs and cats. We decided to see if the explanation to the controversy regarding selective release from the adrenal medulla was that it was due to a species difference between dogs and cats.

The baroreceptor reflex is invoked by a fall in blood pressure in the carotid and aortic arch stretch receptors while the arterial chemoreceptor reflex will result from a fall in the oxygen tension of the blood perfusing the carotid and aortic bodies. Both these reflexes have been shown to induce adrenal medullary secretion and there is some evidence, from Scandinavian and Russian work, that in cats the baroreceptor reflex selectively releases noradrenaline while chemoreceptor stimulation releases a mixture containing a much greater proportion of adrenaline.

We designed a system which enabled us to alter independently baroreceptor tone or stimulate the chemoreceptors in the region of the carotid bifurcation. By giving short discrete stimuli of one or two minutes duration and sampling the effluent blood from the adrenal gland, we compared the noradrenaline to adrenaline ratios in the incremental

releases invoked by the two reflexes. The intensities of the reflex stimuli were adjusted with the objective of obtaining releases of equal magnitude.

It is well known that the mammalian adrenal gland consists of an outer cortex and an inner medulla which are enclosed within the capsule of the gland. The cortex, synthesising steroid hormones, is under humoral control while the medulla has a preganglionic sympathetic innervation and produces noradrenaline and its methylated derivative, adrenaline.

These two tissues of the adrenal gland have quite different embryological origins and classical physiological teaching considers that the control and activity of the two parts are completely independent. However, the internal circulation of the gland is complex and consists of a portal system whereby the cortical effluent blood perfuses the sinusoids of the medulla. There are also through channels, the arteriae medullae, which pass through the outer cortex and carry blood direct from the capsular plexus to parts of the medulla and the central collecting vein.

Only in mammals is there such a close association between the steroid hormone and catecholamine synthesising tissues. Shepherd and West (1951) observed that the ratio of cortical to medullary tissue in the glands of various species directly correlated with the proportion of adrenaline to noradrenaline in the medulla. The cortex, therefore appeared to influence the degree of methylation of noradrenaline to adrenaline. More recently it has been suggested, with much supporting evidence that the continued synthesis of the enzyme which methylates noradrenaline to adrenaline (that is phenylethanolamine-N-methyl transferase - PNMT) requires the high concentrations of glucocorticoid hormones that are delivered to the medullary sinusoids via the portal system from the cortex.

However, we wondered whether this was the full explanation of this complex and intimate relationship between the cortex and medulla. Other vertebrates such as the frog can synthesise adrenaline without the presence of high concentrations of steroids and non-steroid dependent iso-enzymes of PNMT have been reported in mammalian brain tissue.

We thought that the adrenal cortex might also participate in the regulation of catecholamine release. This cortical control of medullary secretion could be mediated through the influence of the concentration of glucocorticoid hormones in the blood perfusing the adrenal medullary cells.

I shall now review the literature on which the previous paragraphs are based. However I will first give a brief historical account of the work on the arterial chemoreceptors, baroreceptors and the adrenal medulla. Then I shall go on to discuss the evidence for the storage of noradrenaline and adrenaline in separate cells as well as the nature of the innervation of the medulla. The work on the various reflexes inducing adrenal catecholamine secretion will be described before considering in detail that done with differential assays which is the basis of the evidence for and against selective release.

Finally I shall discuss the research which prompted us to investigate the influence of the anterior pituitary-adrenocortical axis on adrenal medullary catecholamine secretion.

THE ARTERIAL CHEMORECEPTORS

The systemic arterial chemoreceptors are found in the carotid bodies, lying on each side of the neck immediately rostral to the carotid bifurcation, and in the aortic bodies which are scattered in the tissue between the aorta and the pulmonary artery. They are the receptors in the body which respond to oxygen lack and section of the nerves from the chemoreceptors completely eliminates the normal response to a fall in arterial oxygen tension.

A distinction should be made between the arterial chemoreceptors and the central chemoreceptors. The latter are situated in the floor of the fourth ventricle and the medulla oblongata and maintain a constant arterial PCO_2 by regulating ventilation. Unless I state otherwise, when I use the term chemoreceptor, I am referring specifically to the systemic arterial chemoreceptors.

The existence of the carotid bodies has been known for several hundred years and as a consequence of their far easier access compared with the aortic bodies, they have been used for most arterial chemoreceptor research.

The presence of a ganglion in the region of the carotid bifurcation was first noted by Haller (1743) and then, according to Sigmund Mayer, (1865) by Neubauer in 1772. At first it was named the "gangliolum intercaroticum" and the earliest good description which made reference to its rich sympathetic nerve supply, was given by Lushka in 1862. Sigmund Mayer (1865) confirmed these findings and in the same year Arnold noted the extraordinary vascularity of the carotid bodies which he renamed the

"glomeruli arteriosi intercarotici". J.F. Heymans and C. Heymans (1927) discovered that there were chemoreceptors in the chest sensitive to a lack of oxygen and also to an excess of carbon dioxide. In the following year de Castro (1928) suggested that the carotid bodies in the neck also had a chemo-sensory function and Heymans and co-workers (1930, 1931b) were able to prove this experimentally.

The work of Bouckaert and others (1931) established that by ligating the nerve fibres leaving the carotid sinus in the region between the carotid body and the sinus, the response to pressure changes was removed but changes as a result of chemical stimulation remained unaffected. Comroe and Schmidt (1938) confirmed that the receptors in the carotid region responsible for the hypernoea induced by cyanide, are entirely within the carotid body. Thus the receptors of the carotid sinus were shown to be insensitive to chemoreceptor stimulation and those in the carotid body appeared insensitive to pressure changes. Electrical recordings from the carotid sinus nerve which carried the chemoreceptor fibres, showed that asphyxia or 10% carbon dioxide inspiration increases the frequency of discharge and thus firmly established that these receptor organs are sensitive to chemical changes in the blood (Zotterman 1935).

The peripheral chemoreceptors apparently respond only to three of the four classically described types of anoxia - anoxic, histotoxic and stagnant. They do not appear to respond to anaemic anoxia which explains the absence of hyperventilation in cases of carbon monoxide poisoning or severe anaemia. For their weight the carotid bodies have the highest blood flow of any tissue in the body (2 litres/100 g/min. - Daly et al. 1954) and a very small arteriovenous oxygen difference.

Thus it seems that they respond only to changes in the PO_2 of arterial blood and not oxygen concentration.

A considerable amount of research has been carried out on the arterial chemoreceptors and that prior to 1958 has been excellently reviewed in Heymansand Neil's book "Reflexogenic Areas of the Cardiovascular System". Some of the later research is discussed in the Prolegomenon to "Arterial Chemoreceptors" edited by R.W. Torrance and recently "The Systemic Arterial Chemoreceptors" edited by M. Purves has been published. Anichkov and Belen'kii (1963) have catalogued the pharmacology of the arterial chemoreceptors. Thus, I shall only mention a number of aspects of arterial chemoreceptor physiology which are relevant to this thesis.

1. The chemoreceptors appear to be sensitive to the flow through them in a manner which depends on the PO_2 of the perfusing blood. The explanation of this phenomenon is controversial but a change in flow at a high PO_2 has very little effect compared to the same change at a low PO_2. This property is of importance when one proposes to alter independently baroreceptor and chemoreceptor activity.

2. Hypercapnia potentiates the response of the chemoreceptors to hypoxia. Hornbein and co-workers (e.g. Hornbein and Roos 1963) showed that the response of the chemoreceptors to hypoxia is potentiated by a degree of hypercapnia which has no effect at a higher PO_2. Other workers have published further evidence of the interaction between hypoxia and hypercapnia at a level of the arterial chemoreceptors but it must be remembered that the main component of the whole body response to changes in arterial PCO_2 is mediated via the central chemoreceptors.

3. Chemoreceptor stimulant agents fall into two groups. These are the metabolic inhibitors such as the cyanide ion and the nicotinic drugs (see Anichkov and Belen'kii 1963).

(a) Cyanide was the first stimulant drug used in chemoreceptor studies and it was in 1900 that Pagano noted the hyperventilation and bradycardia in response to intracarotid injections of cyanide and nicotine. However, although he found that the reflex response did not occur if the internal, rather than the common carotid was chosen as the injection site, apparently he was unaware of the existence of the carotid bodies.

(b) Acetyl choline and the nicotinic receptor stimulant drugs also have powerful actions on the chemoreceptors. In fact J.R. Comroe (1960) attributes all the autonomic effects of smoking to the action of nicotine on the chemoreceptors which respond to much lower blood concentrations than are required for significant direct actions on smooth muscle.

4. Hexamethonium abolishes the response of the chemoreceptors to nicotinic drugs but not to hypoxia or cyanide (Douglas 1952)

5. Recently there has been growing evidence for an efferent modulation of chemoreceptor activity. Neil and O'Regan (1971) reported that efferent stimulation of the carotid or aortic nerve increased chemoreceptor blood flow and decreased discharge. Bisco and Purves (1967) have shown that passive limb movements increase chemoreceptor activity via a sympathetic pathway. Joels and White (1968) found that noradrenaline infusions into the carotid blood led to an increased chemoreceptor discharge which was related to the arterial oxygen tension. Thus we entertained the idea that changes in the arterial plasma concentration of noradrenaline could alter the sensitivity of the chemoreceptors by way of its vasoconstrictor action.

Daly and Scott (1963) demonstrated that the primary effects of chemoreceptor stimulation on the circulation are peripheral vasoconstriction and bradycardia with a consequent fall in cardiac output.

However the reflex hypernoea invokes the lung stretch reflex which causes reflex tachycardia and some reflex inhibition of the vasoconstrictor discharge (Daly and Robinson 1968). Thus the overall cardiovascular responses in the spontaneously breathing animal are complex (Korner, Chalmers and White 1967). There is considerable species variation with regard to whether it is the primary or secondary reflex which predominates in the resulting effect of chemoreceptor stimulation on each area of cardiovascular territory.

To my knowledge, although stimulation of the chemoreceptors has been shown to cause increased adrenal medullary secretion, the possibility of any interaction with the lung stretch reflex has not been investigated.

THE ARTERIAL BARORECEPTORS

The baroreceptors are found in the carotid sinus, aortic arch, thyrocarotid junction and the cardiopulmonary area and because of its site, the carotid sinus has been subjected to the most study. They are stretch receptors which are excited by the expansion of the vessel wall and not by the pressure rise itself.

The carotid sinus is a small dilatation at the base of the internal carotid artery. Meyer (1876) noted that this dilatation was associated with a marked thinning of the arterial wall. Schaefer (1877) showed that the sinus was present in all the corpses he investigated and established that it was a normal structure (confirmed by Binswanger 1879).

Work by Hering (1924) showed that stimulation of the central end of the nerve to the sinus caused reflex bradycardia and systemic hypotension and later (1927) he found that sectioning of the nerve abolished all reflexes in response to mechanical stimulation of the sinus.

Following the suggestions of Braucker (1922) and Danielopolu and co-workers (1927), it has been confirmed that the vagus, sympathetic and glossopharyngeal nerves all contributed to the innervation of the sinus. Gerard and Billingsley (1923) described the innervation of the carotid sinus and adjacent carotid body and De Castro (1926, 1928) demonstrated the richness of the sensory innervation to the sinus. Reis and Fuxe (1968) demonstrated the considerable supply of adrenergic nerve fibres which is found in some species. Vasoconstrictor drugs can provoke a big discharge in the afferent fibres from the sinus and this observation has led to much debate as to whether the sympathetic efferent innervation functions as some sort of servo control.

It was shown by Ead, Green and Neil (1952) and a number of other groups that the baroreceptors respond more to pulsatile pressure than to a steady pressure even when the mean pressures are identical.

With a normal blood pressure the baroreceptors exert a tonic inhibitory influence on sympathetic drive to the heart (combined with an increase in vagal activity), the blood vessels (especially muscle resistance vessels) and of course the adrenal medulla.
As the carotid sinus pressure falls, so does this inhibitory influence and it has ceased completely at around 70 mm.Hg, accompanied by a corresponding increase in sympathetic activity and adrenal medullary secretion. (See Heymans and Neil 1958).

THE ADRENAL MEDULLA

INTRODUCTION

One of the earliest descriptions of the adrenal glands was made by Eustachius in 1563 and later Bartholinus (1611) attributed to them the secretion of the "black bile or atrabile". They were named "suprarenal glands" by Winslow. Although this term is appropriate for the human adrenal gland, they are separate from the kidneys in species other than primates and as these do not have an erect posture, the commoner term, adrenal (near to the kidney!) is more suitable. Three centuries after their description by Eustachius, Addison (1855) described the symptoms caused by destructive lesions of the adrenal glands and thus initiated a considerable amount of research on the organs. Brown-Séquard (1856) performed the first experimental adrenalectomies and in the same year Vulpian found that watery adrenal extracts from various species contained a substance which gave a remarkable rose-carmine colour with a number of reagents (e.g. Ferric chloride, iodine, alkali and sunlight). Vulpian also detected this substance in the adrenal vein after death and considered that it was released into the blood stream during life. However, forty years were to pass before the potent pharmacological properties of adrenal extracts began to be investigated which followed the discovery of their pressor effect by Oliver and Schaefer (1894), Cybulski (1895) and Szymonowicz (1896).

In 1897 Abel and Crawford purified and in 1901 Takamine isolated a substance from the adrenal gland which became known as adrenaline. It was the first hormone ever to be isolated or synthesised (Stolz 1904). Independently, Aldrich (1901) also isolated adrenaline and found its empirical formula in 1913.

Also at the turn of the century, Lewandowsky (1899, 1900) and Langley (1901) had both noted that there was a great similarity between the response of various organs to the stimulation of their sympathetic nerve supply and the injection of adrenal extracts. Thus Elliot (1904), who was one of the first people to present the idea of neuro-humoral transmission, suggested that the transmitter substance released at sympathetic nerve endings was adrenaline. However, doubts were laid on this theory by the work of Barger and Dale (1910) and Cannon and Uridil (1921) who showed that the effects produced by sympathetic nerve stumulation were not identical to those produced by adrenaline. On the other hand, Loewi (1921) demonstrated the release of a sympathomimetic substance from frog's heart which was found (Loewi, 1936, 1937) to be adrenaline. Cannon tried to resolve this apparent anomaly by his theory of the two sympathins (Cannon and Rosenblueth, 1933) and he considered that the sympathetic transmitter at the mammalian heart was also adrenaline. However, Raab (1943) and Shaw (1938) showed that the sympathomimetic substance in mammalian extracts was not identical to adrenaline.

Von Euler confirmed their results and went on to show (1946) that a substance extracted from sympathetic nerves as well as from different organs was pharmacologically identical to a compound which was closely related to adrenaline. It had been named, noradrenaline. (The NOR comes from its old German name Stickenstoff-N-Ohne Radikal). Thus von Euler (1948) had identified the sympathetic neurotransmitter and for this work was later awarded a Nobel Prize. About the time he was doing these studies, it was shown both pharmacologically (Holtz et al. 1947) and chemically (James 1948) that the adrenal medulla contains noradrenaline as well as adrenaline. Tullar (1949) then demonstrated the presence of noradrenaline in the commercial adrenaline standards of that time.

Since this work, the presence of noradrenaline in the adrenal glands of various species has been established by many investigators, using a variety of different techniques, and large species differences in the proportion of the two catecholamines have been noted. The presence of noradrenaline in adrenal venous blood was demonstrated by Holtz et al (1947) Bülbring and Burn (1949a)and Kaindl and von Euler (1951). Blaschko (1942) suggested that noradrenaline was the precursor of adrenaline and its conversion to adrenaline was shown by a number of groups e.g. (Holtz and Kroneberg 1949, Bülring and Burn (1949b).

The chromaffin reaction which is used to identify catecholamine - containing tissue, although not differentiating between noradrenaline and adrenaline, was first noted by Henle in 1865. He showed that the cells of the medulla become brown on treatment with chrome salts. Cells with this special affinity for chrome salts were named chromophil by Stilling (1890) and chromaffin by Kohn (1898). Phaeochrome was also suggested as a name by Poll in 1906. Then there followed numerous studies on the nature of the chromaffin reaction and the work by Gerard et al in 1930 resulted in it being accepted for a long time that the colour was due to oxidation products of adrenaline.

However, in 1954 Coupland showed that the compound produced by dichromate is different from that produced by iodate. It appears that following reaction with formol dichromate, chromium is fixed with the formation of chromadrenachrome. Eränkö (1957) then demonstrated the analogous reaction where, following fixation with formol iodate, polymeric iodoadrenochrome derivatives are formed. The chromaffin reaction not only identifies catecholamine containing tissue but many studies have shown that a good correlation exists between the intensity of the reaction and

the concentration of catecholamine estimated in the tissue by chemical assays. A good example is the study of Eränkö and Räisänen (1961) which used photometric measurements of the intensity of the chromaffin reaction.

The adrenal medulla is made up of irregular strands or masses of polygonal cells separated by sinusoidal vessels. However, the notable feature of medullary cells is their cytoplasmic granulation. The granules are evenly distributed in the cytoplasm and localised in them are the sympathomimetic amines of the adrenal medulla. This was shown by Hillarp and Nilsson (1953, 1954) and Blaschko and Welch (1953). Early investigations described two or more different types of medullary cells (Cramer (1916, 1928), Kolmer (1918), Hion (1927) and Ma (1929)). However the significance of the different cell types remained obscure and it was Cramer who concluded that the differences were due to the secretory stage of the cells rather than to their basic properties. This opinion was shared by later investigators such as Bennett (1941), Bachmann (1954) and even Hillarp (1946).

After the discovery of the presence of noradrenaline in adrenal extracts, Bänder (1950) and Eränkö (1951) independently proposed that the presence of two cell types could reflect a difference in the quantitative nature of their catecholamine content. Bänder used classical staining techniques to show two types of cell in the adrenal medulla of the cat, dog and mouse. He called them picrinophil (P) and fuchsinophil (F) and thought them to be related to the production of noradrenaline and adrenaline respectively. Unfortunately, at that time only a little evidence supported this idea. Then Eränkö (1952) using histochemical methods, demonstrated phosphatase positive and phosphatase negative types

of cell in the medulla. These cells were indistinguishable with the chromaffin reaction technique. Furthermore, following treatment with formaldehyde and ammoniacal silver nitrate, the phosphatase negative cells fluoresced under ultra violet light. By microdissection and paper chromatographic separation, he then demonstrated (1954) that the noradrenaline content of the fluorescent cells is far higher than that of the non-fluorescent, phosphatase positive cells. This work was supported (1955) by the correlation found between the number of fluorescent cells and the noradrenaline content in various species.

Richter and Blaschko had shown in 1934 that adrenaline is oxidised by potassium iodate to a coloured compound - (in fact iodoadrenochrome) which is soluble in water. On treatment with this reagent adrenaline cells remain colourless as the bright red coloured compound is leached out by the rinsing solutions. However, the corresponding compound formed from noradrenaline decomposes into a dark insoluble melanin-like derivative (Bu'lock and Harley-Mason (1951). Thus, the adrenal medulla appears as dark staining noradrenaline cells against a background of colourless adrenaline cells. As with the chromaffin reaction, there is a good correlation between the intensity of the iodate colouration and the noradrenaline content of the tissue. In combination with the chromaffin reaction (dichromate), Hillarp and Hökfelt (1954) used this technique to show that there were separate noradrenaline and adrenaline containing cells in the medulla.

The iodate and fluorescent methods were subsequently compared and it was confirmed that they both demonstrate the same type of medullary cell (i.e. that containing mainly noradrenaline.).

As to Bänder's original P and F cells, methods that he himself had proposed in 1954 were used by Eränkö and Palkama (1959) to show that the differential staining Bänder had obtained was dependent on the catecholamine concentration rather than the nature of the catecholamine in the cells.

With autoradiography using radioactive iodine and improved fluorescent methods, Eränkö (1958) obtained further evidence of the segregation of noradrenaline and adrenaline into separate cells.

INNERVATION OF THE ADRENAL MEDULLA

Since the investigations of the late nineteenth century on the functional organisation of the autonomic nervous system, it has been accepted that the splanchnic nerves are the mediators of adrenal secretion.

Jacobj (1891) stated that the splanchnic nerves send fibres to the adrenal gland and Biedl (1897) also suggested that these nerves regulated the secretion of the gland. Jacobj (1892) had already observed that electrical stimulation of the peripheral end of the dog's greater thoracic splanchnic nerve had identical effects on gut contractions as did direct stimulation of the gland itself.

More evidence was Dreyer's (1899) demonstration that electrical stimulation of the splanchnic nerve increased the amount of an active hypertensive substance in the adrenal venous blood. In 1913, Elliot suggested that the chromaffin cells are directly innervated from the primary preganglionic nerves without the intervention of ganglion cells. This was later confirmed by Hoschi (1926), Hollinshead (1936), Swinyard (1937) and others.

The general conclusion of nearly all investigators is that the main innervation comes from the greater thoracic splanchnic nerve but there is also a minor contribution from the lesser splanchnic nerve and the lumbar para-vertebral ganglion (Hollingshead, 1936).

Teitelbaum (1934) suggested the possibility of a vagal contribution but no further evidence in its favour has been obtained.

It was Langley (1896) who demonstrated that the sympathetic ganglion was blocked by nicotine and also showed that the normal pressor effect of

splanchnic nerve stimulation was abolished by previous injections of nicotine. As I will discuss later, although transmission at the adrenal medulla has classically been considered as nicotinic, Feldberg, Minz and Tsudzimura (1934) did demonstrate atropine-sensitive responses after nicotinic blockade.

RELEASE FROM THE ADRENAL MEDULLA

Within a few years of the discovery of the potent properties of adrenal gland extracts, Jacobj (1892) and Dreyer (1899) had shown that electrical stimulation of the splanchnic nerve released a substance with similar properties into the blood. Soon afterwards the effects on the gland of stimuli such as muscular exhaustion (Battelli and Boatta 1902), asphyxia (Elliott 1905) and various centrally acting drugs (Elliott 1912) were described.

During the first half of this century many reflex stimuli and drugs were found to influence the adrenal medulla but, up to 1949, nor-adrenaline was not considered to be an adrenal catecholamine and thus all this foundation work on the nature of the releasing stimuli, can only be interpreted in terms of total catecholamine release.

I shall now describe some of these stimuli with emphasis on those relevant to my thesis. However, at this stage, I shall not consider which, or in what proportions, the two catecholamines are released:-

1. Electrical stimulation of the splanchnic nerve.
2. Electrical stimulation of the Central Nervous System.
3. Stimuli related to hypoxia:-
   - (a) Asphyxia
   - (b) Whole body hypoxia
   - (c) Whole body hypercapnia
   - (d) Selective chemoreceptor stimulation
   - (e) Local action of hypoxia on the gland

4. Stimuli related to a fall in blood pressure:-

   (a) Haemorrhagic hypotension

   (b) Carotid occlusion

   (c) Selective stimulation of the baroreceptor reflex

5. Blood glucose concentration and insulin hypoglycaemia

6. Other reflex stimuli

7. Pharmacological stimulation:-

   (a) Central action

   (b) Chemoreceptor stimulants

   (c) Local action (i) Nicotinic and muscarinic drugs

   (ii) Other drugs

1. ELECTRICAL STIMULATION

The observations of Jacobj and Dreyer have been confirmed many times and the pressor effect of splanchnic stimulation was used by us to tell when we had located the splanchnic nerve in experiments DN1 to DN3.

The following work has been done using differential assays:-

Dogs	Cats
Rapela and Houssay (1953)	Eade and Wood (1958)
Rapela and Covián (1954)	
Klepping (1955, 1956)	
Cier and Klepping (1957)	
Malmejac (See Review 1964)	Calf
Vogt (1965)	Silver (1960)
Mirkin (1961)	
Lund (1951)	

2. CENTRAL NERVOUS SYSTEM - ELECTRICAL STIMULATION

Elliott (1912) suggested that there was a centre participating in the control of adrenal medullary secretion which was situated in the brain stem close to the postulated vasomotor centres. This was based on the observation that the adrenal discharge induced by afferent nerve stimulation was abolished by section of the spinal cord at the T1 segment but not prevented by section at the level of the corpora quadrigemina. On the basis of sectioning and ablation experiments, Cannon and Rapport (1922) concluded that there was a bulbar centre capable of exciting or inhibiting reflexly induced adrenal discharge. (N.B. Nowadays, many authorities consider that the control of the autonomic nervous system is not localised in discrete centres).

Other workers began to search for areas in the brain which on stimulation induced catecholamine secretion and these were found in:-

(a) Bulbar region - Tournade and Malmejac (1932)
Cannon and Rapport (1922)

(b) Fourth ventricle - Cannon and Rapport (1922)
Houssay and Molinelli (1925)
Piqûre (puncture of the Fourth Ventricle) - Kahn (1911)
Carrasco - Formiguera (1922)

(c) Hypothalamus - Houssay and Molinelli (1925)
Magoun, Ranson and Hetherington (1937)
Brauner, Brücke, Kaindl, Neumayer (1951)
Brücke, Kaindl and Mayer (1952)
Grant, Lindgren, Rosen and Uvnäs (1958)
Iremoto (1955)
Redgate and Gellhorn (1953)
Folkow and von Euler (1954)

(d) Mesencephalon - Lindgren (1955)

(e) Medulla oblongata (dorsalateral to the pyramids)
Lindgren, Rosen and Uvnäs (1959)

3. STIMULI RELATED TO HYPOXIA

(a) Asphyxia

A dilation of the denervated iris during asphyxia was described by Budge in 1855 and also observed by Anderson (1903). Elliott (1912) suggested that this phenomenon was mediated by the adrenal glands as no dilatation was observed after adrenalectomy. Between 1910 and 1920 various workers used a number of methods to demonstrate the influence of asphyxia on the gland.

Asphyxia produced by tracheal closure reduced the intensity of the chromaffin reaction in the adrenal glands from dogs, rabbits and monkeys (for comparison one adrenal had been removed before asphyxia) (Kahn 1912, Borberg 1913).

Asphyxia resulted in a higher concentration of catecholamines in inferior vena caval blood samples (taken from above the adrenolumbar veins - Cannon and Hoskins 1911) and in adrenal vein samples (Gley and Quinquand (1917). Anrep (1912) described a vasoconstriction in the denervated hind limb of dogs subjected to closure of the trachea, which was abolished by tying off the adrenal glands.

In 1923, Tournade and Chabrol described their classic technique whereby the adrenolumbar vein of a donor dog is anastamosed to the jugular vein of another dog (recipient was the assay animal) and showed that asphyxia in the donor resulted in a rise in blood pressure in the recipient animal.

Using a similar technique, Houssay and Molinelli (1926) confirmed the results of the latter pair and other workers, using various internal and external bioassays, have generally found that asphyxia resulted in cate-

cholamine release from the adrenal gland. However, Stewart and Rogoff did publish a series of papers between 1916 and 1920 in which they describe testing a variety of preparations but never found any evidence for an adrenal medullary response to asphyxia. This was the case with internal tests such as the denervated iris (1916) and blood pressure (1916) and also external biological assay (rabbit intestine and uterus) of blood collected in a vena canal pocket (1917).

However it is now generally accepted that asphyxia is one of the stimuli to adrenal catecholamine release and further confirmation came from the work of:-

Binet and Lanxade (1936), Herman and Jourdan (1942), Malmejac & Gross (1952), Satō, Inaba & Takahashi (1932)

Asphyxia is a generalised stimulus involving hypoxia, hypercapnia and acidaemia and all of these may act on the arterial chemoreceptors, the central nervous system itself and directly on the gland.

(b) Whole body hypoxia

When this is produced by altering the gas mixture breathed by the animal, the effect on adrenal discharge is similar, although usually less intense, to that of asphyxia. Kellaway (1919) used the denervated iris assay and detected adrenal discharge with oxygen concentrations below about 12%. Houssay and Molinelli (1926) found that an oxygen concentration of less than 11% was required for a measurable response in their adreno-lumbar-jugular anastomosis preparations.

(c) Whole body hypercapnia

Houssay and Molinelli (1926) did not obtain any evidence for an adrenal response to increasing the inspired CO_2 carbon dioxide concentration up to 16%. However Itami (1912) observed a vasoconstriction in the

denervated hind limbs of dogs breathing 10-12% carbon dioxide mixture. Cathcart and Clark (1914) also detected an adrenal response and Tenney (1956) using the denervated nictitating membrane assay found that the carbon dioxide threshold in the cat was 15%.

Not only does hypercapnia directly stimulate the central chemoreceptors in the medulla oblongata but it also potentiates the action of hypoxia on the peripheral arterial chemoreceptors. This interaction between hypoxia and hypercapnia, results in the effect of hypercapnia being, in part, dependent on the arterial oxygen tension and thus hypercapnia is a much greater adrenal stimulus under hypoxic conditions.

(d) Selective stimulation of the Arterial Chemoreceptors

Cyanide was found to be a powerful chemoreceptor stimulant by Heymans et al (1931a)and von Euler and Liljestrand (1937). Many workers have shown that intravenous administration of cyanide induces adrenal medullary discharge although not all of them were aware of the chemoreceptor's peculiar sensitivity to this drug. Its action was considered to be due to a generalised tissue hypoxia which is the case when larger doses than those required for chemoreceptor stimulation are used.

An adrenal catecholamine release in response to potassium cyanide administration was shown by Houssay and Molinelli (1926) using their above mentioned technique. Moller and Stefanson (1937) showed that the hyperglycaemia resulting from hydrocyanic acid administration was abolished by adrenalectomy. Paulet, Cier and Klepping (1957) used sodium cyanide in case the potassium ion was having a direct effect on the adrenal. However, Petropavlovskaya in 1953 had established that the reflex hyperglycaemia was not seen after section of the carotid sinus nerves. She used doses of

around 0.1 mg/kg in non-anaesthesised dogs and decerebrate cats. Later I shall discuss further work by the Russian group under Anichkov (to which Petropavlovska belonged) with reference to differential secretion. Other workers who noted increased adrenal catecholamine release after cyanide administration were Kudo (1931), Suzuki et al (1951), Poshalenko (1955) Malygina (1961), Anichkov et al (1960).

Nicotine has been shown to be a powerful chemoreceptor stimulant (Heymans and Heymans (1927), Heymans et al (1931a)and this action should be considered when assessing the effects of intravenous drug administration.

Much more recently other specific chemoreceptor stimulant drugs have been used to provoke adrenal discharge such as the work done by A. Ungar and his former PhD student D.S.McQueen, using Suberyl Choline.

(e) Local action on the Gland

Finally there is the possibility of a local effect of hypoxia on the gland. A paper by Bülbring, Burn and de Elio (1948) is often quoted as evidence for the direct action of hypoxia on the adrenal gland. They used the 'in situ' dog adrenal which was perfused with the animal's own blood from a closed circuit system containing a Hooker oxygenator. After allowing the preparation to equilibrate for one hour, the gas mixture aerating the blood was changed from oxygen with 5% CO_2 to nitrogen with 5% CO_2. The oxygen-free aeration was continued for a further hour before returning to the original mixture. They did find an increased discharge of catecholamines (output rose to around 4 µg/min) but this response did not start until after half an hour of anoxia. On discontinuing the nitrogen aeration, there was a similar latent period before the catecholamine output began to fall.

Thus there is no evidence here for a local action of anoxia playing a significant part in the response to asphyxia. Bulbring et al did find that the administration of 0.5 - 2.0 mg potassium cyanide to the gland gave

an immediate response which they attributed to the anoxia resulting from the cyanide. However, this amount of potassium chloride also causes a pronounced adrenal release which is due to the action of the potassium ion (e.g. Vogt 1952, 1965).

The paper of Vogt (1952) is often quoted as evidence that anoxia has a direct effect on the glands and is only evidence that authors do not read their references. She was investigating the secretion from the denervated adrenal medulla and in one experiment took one adrenal sample before and one after reducing the blood pressure by severe haemorrhage. She interpreted the two-fold increase in catecholamine secretion rate as being due to the "anoxia" prevailing during haemorrhage. (See p215 for an alternative explanation). This is the sole piece of evidence in this paper that hypoxia has a local action on the adrenal gland.

4. STIMULI RELATED TO A FALL IN BLOOD PRESSURE

(a) Haemorrhagic Hypotension

An increased adrenal medullary output in response to blood loss has been described by many authors e.g. Trendelenburg (1912), Bedford (1917) Stewart and Rogoff (1918), Tournade and Chabrol (1926), Lund (1951), Cier and Klepping (1957), Walker et al (1959).

The response to haemorrhagic hypotension involves the baroreceptor and chemoreceptor reflexes (see Heymans and Neil 1958 p 179) as well as the generalised effects of reduced tissue perfusion and acidaemia.

Others have examined the more discreet stimulus of carotid occlusion.

(b) Carotid Occlusion

The reflex response to carotid occlusion is not solely due to a withdrawal of baroreceptor tone but also has a variable chemoreceptor component due to the reduction in the perfusion pressure of the carotid body.

The anastamoses between the facial vessels, internal carotid and vertebral arteries leads to a rapid recovery of sinus pressure. Various groups have shown that in the dog, although the fall in sinus pressure which results from unilaterial carotid occlusion may be up to fifty percent, it has recovered to within 10-35% of its initial value within half a minute. (Schmidt 1932, Euler and Liljestrand (1936), Chungcharoen et al 1952 and Wang et al 1952). Even though the degree of recovery is less when both common carotids are occluded there is still a good anastamosis in dogs between the internal carotid and maxillary arteries and the vertebral arteries via the circle of Willis.

However, cats very rarely have a patent internal carotid artery (Davis and Storey 1943) and thus there is less recovery of sinus pressure during carotid occlusion in this species. Chungcharoen et al (1952) recorded from chemoreceptor and baroreceptor fibres in the sinus nerve of the cat and found that occlusion of the common carotid on that side gave a great reduction in baroreceptor activity and a small chemoreceptor discharge. If the external carotid was occluded as well, then all baroreceptor activity ceased and there was a heavy chemoreceptor discharge. Thus there is a chemoreceptor component in the carotid occlusion response but it must be remembered that the recovery pressure through anastamotic channels will be far less pulsatile than that before carotid occlusion. Therefore the recovery will be much more effective at reducing the chemoreceptor component by returning carotid body blood flow towards its original level than in restoring baroreceptor tone. The latter receptors respond much more to a pulsatile pressure than to the equivalent mean pressure (Ead, Green and Neil 1952).

Thus the interpretation of the reflex responses from carotid occlusion is variable. It will primarily depend on the initial blood pressure. If the preparation is in a poor state with a low blood pressure it will have no baroreceptor tone and any response can only be due to reduced chemoreceptor perfusion which is likely to be potentiated by the accompanying hypoxia and acidaemia.

In the dog, with a normal or above normal arterial oxygen and blood pressure, the response to occlusion of both common carotids will tend to be small and largely due to the response of the baroreceptors to a great reduction in the pulsatile nature of the sinus pressure. On the other hand, Brauner, Brücke and Kaindl (1950) found that, in unanaesthetised dogs with exteriorised carotids, this stimulus led to a rise in blood pressure which was 50% less when breathing 100% O_2 than when on 7% O_2.

In the cat, the reduction in baroreceptor tone will be much greater but the chemoreceptor component is also increased and very dependent on the arterial oxygen tension.

However, when interpreting the results of carotid occlusion, not only must one consider systemic blood pressure, ventilatory state and species but also note whether or not the preparation was vagotomised. If these nerves are not cut, the aortic baroreceptors will buffer the change in systemic blood pressure/and other reflex responses resulting from carotid occlusion. Finally, the nature of the anaesthesia must be borne in mind as,in cats, chloralose has been shown greatly to reduce the cardiovascular responses to baroreceptor impulses without similarly affecting the chemoreceptor response (Neil, Redwood and Schweitzer 1949). However, we find that a very severe hypoxic chemoreceptor stimulus is required to invoke a significant neural release from the adrenal medulla

(see p.36)

in both cats and dogs. On the other hand the fall in sinus pressure one would expect from carotid occlusion in a cat with a good blood pressure, will provoke a sizeable catecholamine release. Thus I would predict that the catecholamine discharge resulting from carotid occlusion in a pentobarbitone anaesthetised cat would be dominated by the baroreceptor reflex component.

Furthermore, I think that in the chloralose anaesthetised cat the question of which reflex predominates will depend on the balance between the dose of anaesthetic and the state of the preparation.

There is some debate as to whether or not carotid occlusion has an effect at all on adrenal catecholamine output. Aomura (1930) found an increased catecholamine secretion on clamping both carotid arteries as did Hartwick and Hessel (1931) and Von Euler and Liljestrand (1934). As I will discuss in more detail later, the following authors also detected adrenal discharge with the above. Holtz and Schumann (1949) Brauner, Brüche and Kaindl (1950), Brauner, Brüche, Kaindl and Neumayer (1951), Kaindl and Von Euler (1951) and Anichkov et al (1960).

Kaindl and Von Euler (1951) had to cut the vagi in their cats before an adrenal discharge could be observed. A bigger cardiovascular response to carotid occlusion is found after vagotomy for two possible reasons. Firstly, this removes the buffering effect of the aortic arch baroreceptors in response to the rise in systemic blood pressure. Secondly, the inhibitory effect of the lung stretch reflex is abolished. I have already mentioned how the primary vasoconstrictor response to p 14 chemoreceptor stimulation is inhibited by the ensuing reflex hyperventilation and it is possible that adrenal catecholamine release is likewise suppressed. In some of the experiments of Comline and Silver (1966) where they examined the adrenal response to asphyxia in foetal calves, they noticed a sudden drop in secretion if the animal recommenced ventilation.

This may explain why some workers did not detect a significant response to carotid occlusion as was the case in the experiments done on cats by Driver and Vogt (1950). These two did occasionally cut the vagi in the neck but they found that this was without effect on the adrenal release nor did it enhance the blood pressure response. The latter is described as "unusual" by Heymans and Neil (1958-p.64) who consider that the animals may have been depressed by the sodium pentobarbitone anaesthesia.

Cier and Klepping (1957) using dogs also found that carotid occlusion gave only a minimal release. In each experiment they go on to produce hypotension by cardiac slowing due to vagal stimulation and thus I cannot tell if the vagi were cut during the carotid occlusion.

(c) Baroreceptor Reflex - Selective Stimulation

Heymans (1929) was the first to demonstrate satisfactorily that a fall in carotid sinus pressure induced adrenal discharge. The adrenal venous blood from a dog was assayed by means of the degree of splenic contraction in a second dog (using the adrenolumbar-jugular anastomosis). The carotid sinus of the first dog was perfused with arterial blood from a third dog. A fall in sinus pressure resulted in splenic contraction (increased catecholamine output) while a rise in sinus pressure relaxed the splenic vessels (decreased catecholamine output).

However, unless the blood perfusing the carotid sinus was hyperoxygenated there would have been a chemoreceptor component while the sinus pressure was low.

De Schaepdryver (1959) demonstrated that in vagotomised dogs a rise in pressure (to 190 mm Hg) in the vascularly isolated innervated sinus preparation reduced the adrenal secretion nearly to zero.

5. BLOOD GLUCOSE AND INSULIN HYPOGLYCAEMIA

Houssay, Lewis and Molinelli (1924) used an adreno-lumbar-jugular anastomosis to show that insulin hypoglycaemia produced a catecholamine discharge in a donor dog which resulted in hyperglycaemia in the recipient animal.

The observation that insulin hyperglycaemia induces adrenal medullary secretion has been confirmed by Cannon, McIver and Bliss (1924), Abe (1924), Dunér (1954), Goldfein et al (1958) and Wurtman et al (1968).

The depletion of catecholamines from the gland in response to hypoglycaemia is largely prevented by previous denervation (Gohar, 1934, Hillarp 1946).

Dunér (1953) did a very good study on the influence of hypoglycaemia on catecholamine output in cats and found that it decreased adrenal secretion. Furthermore when the head of a recipient animal was perfused via its carotid arteries by blood from a donor, a rise in the donor's blood glucose depressed the adrenal discharge in the recipient.

6. OTHER REFLEX STIMULI

Adrenal catecholamine discharge has been shown to result from the following stimuli:-

(a) Muscular contractions

Small active movements were shown to increase adrenal secretion by Cannon, Linton and Linton (1924), Houssay and Molinelli (1925) and Cannon and Britton (1927).

(b) Centripetal stimulation of an afferent nerve such as the sciatic or brachial plexus or any other painful stimulus e.g. Cannon, Carrasco and Formiguera (1922), Houssay and Molinelli (1925).

(c) Exercise

Battelli and Boatta (1902) found that exercise depleted dog adrenals while Stewart and Rogoff (1922) found a small depletion with severe exercise in cats. I have reviewed elsewhere the evidence for adrenal involvement during exercise in man. (Synapse 1973).

(d) Emotional stimulation, cold and heat have also been shown to be effective adrenal medullary stimulants.

7. PHARMACOLOGICAL STIMULATION

(a) Central action

Since Elliott (1912) observed that morphine and ether, chlorolose and urethane anaesthesia reduced the catecholamine content of cat adrenal glands, the catecholamine releasing actions of anaesthetics have been the subject of much investigation e.g. Elmes and Jefferson (1942) and Vogt (1954).

(b) Chemoreceptor stimulation

A number of drugs release adrenal catecholamines by virtue of their chemoreceptor stimulant action. These have been reviewed by Anichkov and Belen'kii (1963) and include cyanide, nicotine and suberyl choline.

(c) Local action

I have considered the action of nicotinic and muscarinic drugs in my section on selective release. A number of other drugs such as bradykinin, histamine (see discussion), angiotensin and tyramine have been shown, in pharmacological doses, to release catecholamines from isolated adrenal glands (Vogt 1965 and Vane 1969).

However, we have never come across a study similar to that of our own work on the release of adrenal catecholamines by steroid hormones. Established experts in the field such as M. Vogt have told us that they too do not know of any such investigation.

SELECTIVE RELEASE

As mentioned previously, noradrenaline was not considered to be an adrenal catecholamine until 1949 and thus the foundation work on the nature of the releasing stimuli was only in terms of adrenaline. This was the case when W.B. Cannon began to formulate his ideas on the emergency function of the adrenal medulla (e.g. Cannon 1914) and put forward the concept of the sympathetic nervous system as the "flight and fight" mechanism. A single adrenal hormone fitted in well with his view that the sympathetic exerted a diffuse and general influence over the structures which it innervated. He contrasted the sympatho-adrenal system with the parasympathetic which, unlike the former system, dealt with organs separately and not as a group. Cannon talked in terms of the sympatho-adrenal activity evoking widespread changes, such as dilating pupils, accelerating the pulse and constricting splanchnic blood vessels while a reduction in activity permitted these widely scattered organs to resume their former state. "Because of their double innervation the organs under tonic control of the autonomic system can be made to alter the degree of their functional use either as an integrated group, through the sympatho-adrenal system or as separate structures through the direct effects of para-sympathetic impulses". (Cannon 1930's).

Cannon's flight and fight concept profoundly influenced this area of biological thought up to the present day and is still seen in many textbooks as well as being heard at Physiological Society Meetings. Although it is a useful generalisation it has hampered the understanding of the ability of the sympathetic nervous system to respond selectively. However, there is good evidence to show that in a number of reflex responses the sympathetic drive to one organ increases while that to another is

decreasing and thus is not acting as the diffuse and unselective system which Cannon proposed. For example, the primary chemoreceptor reflex involves peripheral systemic vasoconstriction while the sympathetic drive to the heart is withdrawn. However, the increased ventilation invokes the lung stretch reflex which reduces vasoconstrictor tone while at the same time increasing sympathetic drive to the heart. (Daly & Robinson 1968).

Our interest was whether the nervous system could selectively control adrenaline and noradrenaline secretion from the adrenal medulla or were the two catecholamines always released in the same proportions without independent innervation of the two types of chromaffin cell. However, when one considers catecholamine release into the blood it must always be remembered that a considerable proportion of the circulating noradrenaline comes from the adrenergic neurones and not the adrenal gland. Thus the proportion of circulating noradrenaline to adrenaline can be altered by independent changes in adrenergic neurone activity and discharge from the adrenal medulla. (See Appendix I for work on one possible role of noradrenaline as a circulating hormone.) This factor has <u>not</u> been considered in many studies which investigate the influence of variousmanoeuvres on <u>urinary</u> catecholamine excretion. When a change is found in the proportion of noradrenaline to adrenaline excreted, it is often taken as evidence of differential release from the adrenal medulla. Instead, it is merely further proof that sympathetic adrenergic neurone activity and adrenal medullary secretion do not always change in parallel. With regard to the adrenal medulla, once it was established that noradrenaline was released as well as adrenaline, the possibility of independent secretion of the two hormones began to be considered. Most of this research was concentrated at a few centres.

Houssay's group in Buenos Aires had already done much work in the dog on the types of stimuli acting on the adrenal medulla and thus began to assay noradrenaline as well as adrenaline in their experiments. In France, Malmejac and his associates also used dogs and published a considerable number of abstracts in "Comptes Rendus de la Société Biologique".

However, the Scandinavian physiologists did their experiments on cats and U.S. von Euler was their main spokesman. He does not appear to have a very high opinion of the French workers. In his book "Noradrenaline" (1956) he refers to only one of their publications and then only with regard to their results on resting secretion. He argued in favour of the view that various physiological stimuli could independently release noradrenaline or adrenaline from the adrenal medulla. On the other hand, Malmejac did not believe that independent release occurred.

They both put forward their views in a number of papers and reviews and each tended to play down the publications of the other groups.

They presented opposing papers at the XXI International Congress of Physiological Sciences in Buenos Aires 1959 and certainly do not appear to have been great friends. However, although Malmejac wrote a Physiological Review in 1964, his work does appear to have been largely ignored. Although this will, in part, be due to von Euler's prestige, Malmejac's case has not been helped by his own habit of publishing work in the form of numerous small and repetitive abstracts in French, in a relatively inaccessible journal.

Over the past twenty years various authors have argued for and against independent adrenal catecholamine release but in all that I have read, no one has made more than a passing reference to the possibility

of a species difference between the Dog and the Cat. Nearly all the work pertinent to selective release has been performed in these two species. Although many writers have discussed a good part of the work in order to support one idea or the other, as far as I know no one has tried to explain the controversy by examining the published data to see if selective release occurs in one species but not in the other. Furthermore, no one laboratory has tried the same set of reflex stimuli in both dogs and cats.

On examining the literature I found that in the dog, the data suggests that whatever the nature of the stimulus to the adrenal gland, noradrenaline and adrenaline are released in about the proportion of 1 to 4. Any claims for independent secretion of one catecholamine (always adrenaline) are usually based on comparisons with the catecholamine proportion in very low control outputs and one finds the 1 to 4 ratio holds for the composition of the releases. Most published data gives the proportion of noradrenaline in the releases as between 15-20% except for Malmejac's group which find it to be around 5% - a figure they obtain with both biological and chemical assays.

On the other hand the results obtained from Cats appear to show that the adrenal medulla has the capacity to release independently noradrenaline and adrenaline. This evidence comes from work with hypothalamic stimulation (von Euler), changes in blood glucose (Dunér), chemoreceptor reflexes (Anichkov), etc. as well as more recent work by Fuerstein and Gutman.

I shall now examine the research in more detail.

Papers discussed with reference to Selective Release

Dogs

1. Rapela and Houssay (1952)
2. Malmejac (1964 Review)
3. Cier and Klepping (1957)
4. Lund (1951)
5. De Schaepdryver (1959)
6. Mirkin (1961)
7. Goldfien and Ganong (1958)
8. Wurtman et al (1968)

Cats

1. Holtz and Schumann (1948)
2. Brauner, Brücke and Kaindl (1950)
3. Kaindl and Von Euler (1951)
4. Brücke, Kaindl and Mayer (1952)
5. Von Euler and Folkow (1953)
6. Folkow and Von Euler (1954)
7. Dunér (1953, 1954)
8. Redgate and Gellhorn (1953)
9. Anichkov and Belen'kii (1963)
10. Grant, Lindgren, Rosen, Uvnäs (1958)
11. Eade and Wood (1958)
12. Fuerstein and Gutman (1971)

DOGS

1. Rapela and Houssay (1952)

Most of the Argentinian work was done by B.A. Houssay and C.E. Rapela who investigated the effect of the following stimuli on the secretion of adrenaline and noradrenaline by the adrenal gland:-

1. Electrical stimulation of the greater splanchnic nerve (Rapela and Houssay 1952, Rapela and Covián 1954).
2. Nicotine - Intravenous administration (Rapela and Houssay 1952).
3. Asphyxia (Rapela and Houssay 1952)

They claim that both electrical stimulation and nicotine preferentially release adrenaline, while asphyxia does not produce any change in the proportion of the two catecholamines.

However, after examination of their figures, I feel that their conclusion is not justified.

I calculated from their data that the percentages of adrenaline (mean $\pm$ S.E.M.) in the output during the various stimuli are as follows:-

1. Electrical stimulation (with Havard inductorium) = $79.5 \pm 3.7\%$
2. Nicotine (denervated gland) = $83.8 \pm 4.6\%$
3. Asphyxia = $85.6 \pm 4.3\%$

Thus there is no evidence, in this work, for what we define as selective release. However, I shall now comment on their experiments and conclusions.

1. Electrical Stimulation

They performed two series of experiments. The first used a Harvard inductorium and gave an output containing 79.5 ± 3.7% (n = 17). N.B. I did not attempt to calculate increments as the control outputs were usually < 2% of that during stimulation. The second series used a Grass model 3C stimulator and gave outputs containing 76 ± 6, 65 ± 6 and 73 ± 4% at 10, 40 and 160 pulses per second respectively. Only the lowest rate is physiological (see Celander, 1954 p.7&19). The reduction in adrenaline at the higher rates may be explained by exhaustion of stores; thus the adrenaline proportion will fall due to its slower rate of synthesis.

2. Nicotine

Denervated glands were used and the doses gave outputs between 10 and over 200 times the controls. Here the adrenaline proportion in the output was 83.8 ± 4.6%.

3. Asphyxia

This gave a thirty fold release with 85.6 ± 4.3% adrenaline in the output.

Thus the three stimuli released the same proportion of noradrenaline to adrenaline and the reason why Rapela and Houssay considered that two of the stimuli selectively released adrenaline while the other did not, was that they compared them with two different types of control percentage. For the asphyxia experiments they used the control output from the innervated gland which they found to contain 86.8 ± 5.7% adrenaline and thus concluded that there was no preferential secretion with asphyxia.

However, when they considered nicotine and electrical stimulation, they used the control output from the denervated gland which contained 59.7 ± 9.0% (nicotine experiments)and 69.9 ± 4.2% (electrical stimulation experiments).

The only explanation that I can offer for this observed fall in adrenaline proportion after nerve section is as follows:-

At the reduced adrenal outputs which are usually seen after section and tend to give the low adrenaline percentages, the circulating noradrenaline in the blood will contribute significantly to the adrenal output. For example, consider a 10 Kg dog (they used animals of about this weight) with a control output of 4 ng/Kg.min which contains 75% adrenaline:-

The noradrenaline output from the gland = 10 ng/min

With an arterial blood noradrenaline of 0.5 ng/ml, which is very likely in the haemorrhagic experimental animal, and an adrenal blood flow of 8 ml/min:-

The delivery of noradrenaline to the gland = 4 ng/min

∴ Actual adrenal noradrenaline output = (10 - 4) = 6 ng/min

∴ Adrenaline proportion of circulating catecholamines:-

$$\text{Adrenaline} = \frac{30}{36} \times \frac{100}{1} = 83\%$$

Thus circulating noradrenaline could explain the lower adrenaline proportion in the outputs from denervated glands. A drawback to my explanation is the small difference between the denervated and pre-asphyxia control outputs. Furthermore, there was no change in output on denervation in six out of the eleven experiments.

However, their control outputs are very small and even assuming that their assay (rat uterus and dog blood pressure), was adequate over a two hundred range in total amount, I do not think it is justified to claim that the preferential secretion of adrenaline occurs. I may appear to have laboured the point but Rapela and Houssay's conclusion is often quoted in favour of selective release

2. Malmejac (Review 1964)

From the work that he and his group did on dogs, Malmejac concluded that, whatever the stimulus, adrenaline and noradrenaline were released in the same ratio. This multitude of publications is very adequately reviewed in his 1964 article "Activity of the Adrenal Medulla and its Regulation" (Physiol. Rev. 44,186). I shall not waste space attempting to discuss them all in detail.

Their more useful work was done on the irrigated "in situ" adrenal gland which involved perfusing the adrenal region of the aorta with blood from a donor. Bioassay or later fluorimetry, was used to assay the catecholamine secretion.

An example of the sort of results they obtained is:-

Stimulus	Catecholamine Output ng /Kg. min.	Noradrenaline %
Hypotension	1024	3%
Asphyxia	702	10%
Anoxia	351	10%
Acetyl Choline i.v.	633	4%
Nicotine i.v.	2366	12%
Splanchnic nerve	2854	2%

The odd thing about all their results is that they always found a low noradrenaline percentage no matter which assay they used. (Perhaps the donor dogs had very high circulating corticotrophin levels-see p.237).

The noradrenaline percentage rose on sectioning the nerve but this can be explained by the contribution of circulating noradrenaline from the donor.

3. Cier and Klepping (1957)

Cier and Klepping looked at a number of secretory stimuli. Their published results are very variable and give no consistent evidence of selective release with cyanide (Paulet, Cier and Klepping 1957) or various forms of hypotension (Klepping and Cier 1957). No doubt as a consequence of the larger releases, their assay appears more reliable when splanchnic stimulation was used and gave on average 20-30% noradrenaline. Increasing the frequency of stimulation (3, 20 and 40) gave no convincing shift in ratio (Klepping 1956).

4. Lund (1951)

More reliable work on the influence of splanchnic nerve stimulation is that of Lund (1951). He assayed fluorimetrically the adrenal venous effluent from chloralose anaesthetised dogs during electrical stimulation (50/sec) and haemorrhage. He observed no change in noradrenaline to adrenaline ratio with either stimulus.

5. De Schaepdryver (1959)

The trihydroxyindole fluorimetric assay was also used by De Schaepdryver whose work is sometimes quoted in favour of selective release in the dog. He quotes mean values without giving standard errors or any

idea of the number of determinations involved. In other publications he does give this data and, as he did over thirty different procedures in only 48 dogs, it is likely that each procedure was done in only one or two animals. Thus, as the noradrenaline proportions in the resting and incremental outputs during carotid occlusion, acetyl choline, nicotine and insulin administration are between ten and forty percent, there is no convincing evidence here for selective reflex release. Furthermore, he himself states that there is no significant change in noradrenaline to adrenaline ratio on carotid occlusion and his claims for a selective effect on secretion are with reference to the action of the drugs Reserpine, Iproniazid and P-286.

6. Mirkin (1961)

Mirkin (1961) used direct bioassay (Cat blood pressure and chick rectum) to investigate the content of adrenal venous sample collected during stimulation of the splanchnics at 1-20 pulses/second. He found a linear relation between frequency and output up to 10 hertz with no change in the noradrenaline percentage released. However, he claims that the noradrenaline percentage fell by 10-20 percent at 20 hertz and cooling of the nerve increased noradrenaline percentage by a similar amount.

7. Goldfien and Ganong (1958, 1960)

Wise, Goldfien and Ganong (1960) publish results for output from the right adrenal gland in response to haemorrhage:-

	A	NA	NA%
Resting output	93 ± 25	17 ± 5	15%
5 minutes after haemorrhage	209 ± 83	10 ± 4	5%
20 minutes after haemorrhage	276 ± 98	12 ± 5	4%

(ng/min)

Their standard errors for eight experiments are very large but there is definitely no evidence of selective release of noradrenaline in response to haemorrhage. Although their results are rather erratic the work of Goldfien et al (1958) provides further evidence that during insulin induced hypoglycaemia, noradrenaline and adrenaline are released in a 1 to 4 ratio.

8. Wurtman et al (1968)

Some recent work done by Wurtman et al (1968) provides good supporting evidence for our view that catecholamines are always released from the adrenal medulla of the dog in the proportion of 1 to 4 (noradrenaline:adrenaline). These are the workers who have investigated the influence of the pituitary-adrenocortical axis on the methylating enzyme and thus are experienced at differential assay. They find that the increase in adrenaline output after insulin is paralleled by a rise in noradrenaline secretion and the adrenaline proportion in the release is 82 ± 4%.

C A T S

1. Holtz and Schumann (1948)

They found that in the cat, carotid occlusion produced a contraction of the spleen and an increase in blood pressure but had no effect on blood sugar or peristalsis. However, a dose of adrenaline which gave a comparable degree of splenic contraction also arrested peristalsis while the corresponding dose of noradrenaline did not affect gut motility. The splenic response to carotid occlusion was abolished by adrenalectomy and thus the authors concluded that this reflex stimulus releases noradrenaline from the cat adrenal gland.

A later paper (Holtz, Engelhadt, Greeff and Schumann, 1952) confirmed their earlier finding that carotid occlusion releases mainly noradrenaline.

They employed barbituate anaesthesia and it is probable that in these tests the baroreceptor reflex was the main component of the adrenal response to carotid occlusion. Using the cat blood pressure and chicken rectum to assay adrenal blood samples, they detected a resting output containing 78% noradrenaline (72-86%).

Carotid occlusion gave a two fold increase in output and the increments contained 68% noradrenaline.

On the other hand, direct stimulation of the splanchnic nerves gave ten to thirty fold increases which contained only 10% noradrenaline.

2. Brauner, Brücke and Kaindl (1950)

In these experiments the cats were anesthetised with chloralose and they found that the catecholamine release in response to

carotid occlusion was abolished by breathing 100% O_2. Repeated carotid occlusion gave rise to effects indicating an increasing proportion of adrenaline in the output. In a second paper (Brauner, Brücke, Kaindl and Neumayer, 1950) which is based on a delightful assay of adrenal venous samples, involving the pilomotor action of catecholamines on the hairs of the cat's tail (and the isolated rabbit jejunum), they again state that carotid occlusion released a large proportion of adrenaline.

3. Kaindl and Von Euler (1951)

Kaindl joined forces with Von Euler and the following year published a paper criticising the first technique used by his former colleagues. This was on the grounds that their assay depended on the animal's own blood pressure and intestinal volume which were both under the influence of adrenergic innervation. Kaindl and Von Euler worked on vagotomised cats anaesthetised with chloralose and used the cat blood pressure and chick rectal caecum to assay their samples.

The resting secretion was on average 73 ng /kg. min and contained 77% noradrenaline (60-89%). With carotid occlusion, catecholamine output rose to a mean of 230 ng /kg. min ; although there was only a slight rise when the vagi were intact.

The increments contained 75 $\pm$ 3% noradrenaline and in two out of the four experiments, there was a tendency for the noradrenaline proportion to fall as the experiment progressed.

The important question is whether carotid occlusion was invoking mainly a baroreceptor or a chemoreceptor reflex. The resting blood pressure was usually over 150 mmHg. and presumably the preparation

was in a good state with a normoxic arterial PO_2. The blood pressure response to the tests was good (usually over 250 mmHg) except when the vagi were intact and thus it appears that the depressant effect of chloralose anaesthesia (50 mg./kg) on the baroreceptor reflex (see p.106) was not sufficient to inhibit markedly the aortic baroreceptor reflex. Furthermore, resting system blood pressure was over 150 mmHg and as it rose during the reflex test to over 250 mmHg, carotid sinus pressure would have been unlikely to fall much below 80 mmHg. Hence, I expect that the chemoreceptor component in the adrenal response was small.

4. Brücke, Kaindl and Mayer (1952)

Kaindl then went back to his old friends and investigated the action of hypothalamic stimulation on secretion from the cat adrenal medulla. Brücke, Kaindl and Mayer (1952) found that hypothalamic stimulation released equal amounts of adrenaline and noradrenaline while the resting secretion was 90% noradrenaline. (Biological assay of adrenal venous samples).

5. Von Euler and Folkow (1953)

Von Euler then worked with Folkow and compared carotid occlusion with afferent stimulation of the sacral and brachial plexus. Carotid occlusion again released about 70% noradrenaline (60-90%) but in every experiment, afferent nerve stimulation always released a greater proportion of adrenaline although the composition of the mixture varied between experiments from 10 to 75% noradrenaline. In each out of eleven experiments, nerve stimulation produced twice the adrenaline output, but a similar noradrenaline output as carotid occlusion. (Biological assay of adrenal venous samples).

6. Folkow and Von Euler (1954)

The following year they (Folkow and Von Euler 1954) used a Horsley-Clarke instrument to stimulate in the region of the hypothalamus. Again using chloralose-anaesthetised cats and the cat blood pressure/chick rectal caecum differential assay, they found brain areas which selectivaly released adrenaline, noradrenaline or a variable mixture of the two.

They did sixteen successful experiments and although they do not present full details of their results, this paper is one of the most quoted in favour of selective release. (It is written in English). At the end of nine experiments, they injected (i.v.) 1-2 mg /kg. of nicotine which as well as having a direct action on the gland, must have evoked a maximal chemoreceptor discharge. The proportion of adrenaline in the adrenal output nearly always increased relative to the preceding "resting" secretion, but the composition varied from 20-86% noradrenaline.

7. Dunér (1953, 1954)

Dunér (1953) did some of the best described work on selective release from the cat adrenal gland. Using pentobarbitone — anaesthetised cats and the cat blood pressure/chick rectal caecum assay, he investigated the influence of hyperglycaemia on adrenal secretion.

In ten cats with a resting secretion of 14 $\pm$ 4 ng /kg. min adrenaline and 70 $\pm$ 12 ng /kg noradrenaline (86% noradrenaline), glucose infusions induced varying degrees of hyperglycaemia and selectively depressed adrenaline secretion.

Thus adrenaline output (plotted as a percentage of the initial resting output) was linearly related to blood glucose and fell to about zero at a glucose concentration of 500 mg/100ml. On the other hand

noradrenaline did not fall below 70% of its initial output and thus the noradrenaline proportion in the mixture rose with increasing glucose concentration.

Cross perfusion and other experiments confirmed that the blood glucose concentration was acting through a central mechanism.

In eight cats, Dunér compared the composition of the resting secretion (12 ± 5% adrenaline) with the content of the gland itself (45 ± 2% adrenaline) and found no correlation.

In a further paper, Dunér (1954) describes six experiments (again pentobarbitone anaesthesia) in which insulin hypoglycaemia resulted in selective adrenaline release. Adrenaline output would increase/by usually about ten fold while noradrenaline output never rose by more than 25-50%.

It is interesting to note that Hökfelt (1951) showed that, in rats, large doses of insulin almost completely depleted the adrenal glands of adrenaline, while noradrenaline content changed much less. Similar results were obtained by Outschoorn (1952).

Dunér is claimed by a number of authors (e.g. Von Euler, 1956) to have investigated the influence of hypoxia and hypercapnia on catecholamine release. However, a reference is never given and I have been unable to find this publication.

8. Redgate and Gellhorn, 1953

Another selective release classic, is the paper by Redgate and Gellhorn (1953). Their work is often claimed to show selective release of adrenaline by asphyxia (chloralose anaesthesia) and adrenaline or noradrenaline by hypothalamic stimulation (pentobarbitone anaesthesia).

They used the denervated nictitating membrane and the denervated spleen volume as an 'in vivo' differential assay of circulating catecholamines. The former preparation assayed adrenaline while the spleen responded to both catecholamines but unfortunately was relatively less sensitive. Close examination of their published graphs confirms that if noradrenaline was also released during asphyxia, it could only have been in smaller quantities to the amounts of adrenaline detected. However, they were apparently unaware that the release of noradrenaline into the circulation from adrenergic nerve endings usually predominates over that from the adrenal medulla. Thus the detection, during hypothalamic stimulation, of an increase in circulating noradrenaline without a corresponding rise in adrenaline, is more likely to be evidence of adrenergic neurone activation than selective noradrenaline release from the adrenal medulla.

9. Anichkov et al 1960

In Russia, Anichkov's group carried out a considerable number of investigations on the reflexes invoked by chemoreceptor stimulation and this included adrenal catecholamine release (Anichkov and Belen'kii, 1963, p. 34-41).

Experiments were carried out on decerebrate cats and compared chemoreceptor stimulation resulting from an intracarotid injection of potassium cyanide with that from carotid occlusion.

Their assay was rather unusual, consisting of the 'in vivo' denervated leg (reduction in limb volume determined by plethysmography) and the denervated, cocaine-sensitised, nictitating membrane. They used a dose of cyanide which gave a change in limb volume to match that

from carotid occlusion. However, this dose resulted in greater contraction of the nictating membrane. Thus:-

Limb volume: $\frac{\text{Potassium Cyanide}}{\text{Carotid Occlusion}} = 1.06 \pm 0.06$

N.M. Contraction: $\frac{\text{Potassium Cyanide}}{\text{Carotid Occlusion}} = 1.70 \pm 0.18$

They claim that noradrenaline produces the greater reduction in limb volume and adrenaline produces the greater contraction of the nictitating membrane. We must assume that they checked this by injecting standards into their preparations.

Thus it would appear that chemoreceptor stimulation releases a mixture with a higher proportion of adrenaline than carotid occlusion. (Anichkov, Malyghina, Poskalenko and Ryzhenkov, 1960).

10. Grant et al 1958

Grant, Lindgren, Rosen and Uvnäs (1958) topically stimulated the area of the hypothalamus in cats which activates the atropine-sensitive vasodilator fibres to skeletal muscle. They found selective release of adrenaline from the adrenal medulla during such stimulation.

They investigated fourteen cats under either chloralose/urethane, urethane or Dial anaesthesia and found that the adrenaline output increased, on average, over fourfold, while there was often no noradrenaline increase at all (in fact, an average increase of 84%). Under Dial anaesthesia the selectivity was most marked, with four to sixty fold increases in adrenaline and no change or a fall in noradrenaline output.

The mean resting output of 250 ng /kg. min consisted of between 67 ± 4 to 98 ± 0.3% noradrenaline depending on the nature of the anaesthesia. The increment on hypothalamic stimulation was on average 420 ng /kg. min and contained a mean of 38% noradrenaline.

I will discuss later their results concerning the influence of anaesthesia on the composition of the resting secretion.

11. Eade and Wood (1958)

This pair of workers examined the composition of the catecholamine mixture released during splanchnic stimulation (cats anaesthetised with chloralose) using a fluorimetric assay very similar to ours. Electrical stimulation released between 40-50% noradrenaline and there was a tendency for the noradrenaline proportion to fall with the three successive stimulations:

i.e. 48 ± 3% 42 ± 3% and 39 ± 4%

As the glands contained 37 ± 3% noradrenaline, it appears that electrical stimulation releases a mixture with a similar composition to that found in the medulla.

12. Fuerstein and Gutman 1971

The most recent evidence in favour of selective catecholamine release in the cat comes from Israel.

Fuerstein and Gutman (1971) used pentobarbitone anaesthesia and assayed their adrenal venous plasma samples fluorimetrically.

The stimuli employed were haemorrhage, hypoglycaemia and hypothermia and I have extracted some relevant information from their table of results:-

	Total Catecholamine Output (ng /kg. min)	% Adrenaline
Resting output	5 ± 1 to 6 ± 1	79 ± 3%
Acute haemorrhage	67 ± 18	32 ± 6%
Hypoglycaemia	44 ± 8	68 ± 5%
Hypothermia	No release	40 ± 7%

Thus hypotension, with its baroreceptor and chemoreceptor reflex components, released a mixture containing predominantly noradrenaline (68%) while hypoglycaemia released mainly adrenaline (32%).

Hypothermia appeared to change the proportion of catecholamines in the resting output.

SUMMARY OF THE WORK ON CATS

All those workers who have examined the influence of various reflex stimuli on the release of adrenaline and noradrenaline from the adrenal medulla of the cat, have found evidence of selective release.

Carotid occlusion and haemorrhage, which probably exert their main effect on the adrenal medulla via the baroreceptor reflex, give rise to a secretion containing about 70% noradrenaline (i.e. Kaindl and Von Euler (1951), Von Euler and Folkow (1953), Fuerstein and Gutman (1971) with supporting evidence from Anichkov's group).

There is good evidence that hypoglycaemia releases predominantly adrenaline (Duner, 1954, Fuerstein and Gutman, 1971) and from Duner's work (1953, 1954) it appears that blood glucose exerts its reflex effects only on adrenaline secretions.

Asphyxia and cyanide-induced chemoreceptor stimulation seem to preferentially release adrenaline (Redgate and Gellhorn (1953), Anichkov et al (1960)) but this work depended on less reliable assays.

Stimulation in the region of the hypothalamus has been shown by three groups to release adrenaline selectively (Redgate and Gellhorn, (1953), Folkow and Von Euler (1954) and Grant et al (1958)).

Folkow and Von Euler (1954) also found stimulation points in the brain which selectively released noradrenaline or varying proportions of the two catecholamines.

NICOTINIC AND MUSCARINIC RECEPTORS IN THE ADRENAL MEDULLA

The transmission from the preganglionic nerve endings to the chromaffin cells of the adrenal medulla is classically regarded to be of the nicotinic (ganglionic) type. However, in 1934 Feldberg, Minz and Trudzimura had found evidence of a potential muscarinic component. By using the pressor effect as an index of catecholamine release, they observed that a large blocking dose of nicotine did not appreciably alter the response of the cat adrenal medulla to acetyl choline. If atropine was given as well as nicotine the response was blocked but the same dose of atropine alone did not convincingly reduce the response to splanchnic stimulation and so atropine did not appear to be acting through a local anaesthetic effect.

However, muscarinic transmission only appeared important during nicotinic blockade and these observations were largely forgotten until the work of Douglas and Poisner (1965). These two were engaged in a series of experiments with the aim of investigating the part played by calcium in catecholamine release and were using isolated cat adrenal glands perfused with Locke solution.

They decided to re-investigate Feldberg's observation and not only did they confirm that muscarinic stimulation released adrenal catecholamines but they also showed that the mixture released contained less than 20% noradrenaline while that in response to nicotine contained over 50% noradrenaline. The mean percentage of the total catecholamine appearing as noradrenaline was only 4% with pilocarpine and 16% with muscarine while acetyl choline and nicotine released 58% and 55% respectively.

Rubin and Miele (1968) followed up this work in cats and using the same technique, found that, although high doses of nicotine (1.2×10^{-5} M) released approximately equal amounts of the two catecholamines, lower doses (1.2×10^{-6} M) gave outputs containing about 70% noradrenaline. Doses of acetyl choline which gave outputs of comparable magnitude to the lower doses of nicotine still released the catecholamines in equal proportions.

Further evidence comes from the work of Butterworth and Mann (1957) who investigated the action of serial injections of acetyl choline in atropinised (6 mg /kg) chloralose anaesthetised cats which had had the right adrenal gland removed. These injections, of the order of 2 mg intravenously, gave very reproducible pressor responses which slowly declined over the course of the experiment (up to 100 doses). The pressor effect was abolished by removal of the other gland but was still seen after splanchnic nerve section and thus was not due to chemoreceptor stimulation. They found that acetyl choline and other choline esters depleted the noradrenaline and adrenaline in the glands by the same factor and so the noradrenaline percentage in the depleted gland was the same as that in the right (control) gland (mean = 64% noradrenaline). Although there was much variation between animals (35-90% noradrenaline), within each experiment the percentage of noradrenaline in the adrenal venous effluent (mean = 62% noradrenaline) was always the same as that in the gland.

Thus it appears that in the atropinised animal, acetyl choline releases the same proportion of catecholamines as found in the gland and this figure of 60-65% noradrenaline is compatible with the results obtained with acetyl choline and the higher doses of nicotine in the work of Douglas and Poisner (1965) and Rubin and Miele (1968).

On the other hand, Kayaalp and McIsaac (1968) used dog glands and reported that nicotinic stimulation released a mixture containing 21% noradrenaline. The output in response to methacholine consisted on average of 40% noradrenaline but due to the scatter in their results, the difference between the two types of stimulation is not significant.

However, once again there appears to be a species difference between the dog and the cat with respect to selective activation of noradrenaline and adrenaline release. In the dog, both nicotinic and muscarinic stimulation released similar proportions of the two catecholamines while in the cat, nicotinic drugs had much more of an effect on noradrenaline release than did muscarinic stimulation.

I must stress that I realise that a difference in distribution of the two types of receptor cannot explain, by any simple mechanism, selective reflex release in the whole animal.

EVIDENCE FOR THE PARTICIPATION OF THE ANTERIOR PITUITARY-ADRENOCORTICAL AXIS IN THE REGULATION OF ADRENOMEDULLARY SECRETION

Influence of the Adrenal Cortex on Medullary Catecholamine Secretion

Although it is generally accepted that the adrenal cortex and medulla function independently, there is growing evidence that the cortex does exert a significant influence on the medulla.

First, there is the existence of the complex internal circulation of the gland with a portal system carrying blood rich in steroid hormones to the medulla.

Secondly, there is the work of Shepherd and West (1951) and Coupland (1953) which drew attention to the correlation between the cortex to medullary size ratio and the percentage of adrenaline in the glands of various mammalian species. More recently Pohorecky and Wurtman (1971) have shown in the rat that the high concentration of glucocorticoids delivered to the medulla from the cortex is essential for the maintenance of the enzyme, Phenylethanolamine-N-methyl transferase (PNMT) which methylates noradrenaline to adrenaline.

Thirdly, there is a little evidence from studies on adrenal gland catecholamine content that corticotrophin administration results in the depletion of adrenaline but not noradrenaline.

Finally, the results published by Wurtman, Casper, Pohorecky and Bartler (1968) in support of their above mentioned theory also contain some good evidence that corticotrophin presumably by acting through glucocorticoid secretion, modulates the release of catecholamines in response to neural stimuli.

Blood Supply to the Adrenal Gland

This is very thoroughly reviewed by R.E. Coupland in his book "The Natural History of the Chromaffin Cell" (1965, p.113-119) which is well worth reading. He not only describes, with an awareness of cortico-medullary relationships, the details of the anatomy of the vasculature but also discusses some interesting work on the factors which affect intra-adrenal blood flow.

R.G. Harrison and M.J. Hoey produced a monograph entitled "The Adrenal Circulation" (1960) which also contains a good description of the gland's vasculature.

Multiple small adrenal arteries, derived from the nearby large arteries - (the balance of which depends on species), converge on the gland and in man there are fifty to sixty of these small vessels. They arborize in the peri-adrenal connective tissues and form a capsular plexus in the dog or a subcapsular plexus in the cat and rat. Capillary vessels, supplied by arterioles from the plexus, pass centripetally between and around the columns of cortical cells. A few arterioles pass deep into the cortex before breaking up into capillaries (arteriae corticis).

The blood supply to the medulla is from two main sources. These are the capillary channels of the cortical zona reticularis which become the sinusoids of the medulla. Also the arteriae medullae pass through the cortex from the "capsular" plexus before breaking up into capillary channels which form a network around adjacent chromaffin cells. Capillary blood vessels derived primarily from the arteriae medullae anastomose with each other and the branches of the venous tree (Flint 1900). Thus not all the blood flowing through the arteriae medullae enters the cortico-medullary sinuses as it can also pass directly to the

central vein through the venae medullae.

The above description is taken from Coupland's book but most of the points were first noted by Flint (1900) who worked on dog glands.

It is very interesting to note that in ungulates such as the sheep and the cow, the regions of cells containing solely noradrenaline are supplied almost entirely by the arteriae medullae. However, the adrenaline storing cells are arranged in palisade form along the cortico-medullary venous sinuses and have well defined arterial and venous poles.

Symington (1962) has described bundles of smooth muscle flanking the orifices of the cortico-medullary venous sinuses. He suggests that contraction of this muscle will obstruct blood flow from the venous sinuses without interfering with that from the central vein. The anatomy of the musculature of the adrenal vessels is complex and not understood but one wonders if it could alter the distribution of flow between the cortico-medullary sinusoids and the arteriae medullae. This would change the concentration of glucocorticoids bathing the medullary cells.

Dependence of Methylation on the Cortex

When Shepherd and West (1951) surveyed the percentage of adrenaline in the adrenal medulla of various mammalian and avian species they found a good correlation with the ratio of the volume of cortical to medullary tissue. In species where this ratio is high such as in the cavy and rabbit, the medulla contains almost exclusively adrenaline while in those with a lower ratio, (e.g. cat, dog and man) a greater percentage of noradrenaline is found.

Moreover, the medulla of foetal mammals contains very little adrenaline and the adrenal content of this catecholamine rises sharply after birth in parallel with a period of marked cortical development. (Cavy and rabbit - Shepherd and West, 1951; Human - West, Shepherd and Hunter, 1951; Calf - Holton, 1951).

The dependence of methylation on the cortex only appears to be the case in mammals. Other vertebrates which have separate steroid and catecholamine synthesising tissues, have chromaffin glands containing a large proportion of adrenaline (Shepherd, West and Erspaner (1953) e.g. 20-67% adrenaline in various species of fish).

However, Coupland (1958, 1959) claims to have transplanted adult rat adrenal medullary tissue to the orbital cavity and found that these transplants had a normal adrenaline to noradrenaline ratio despite being deprived of the majority of cortical cells.

This latter work is hard to reconcile with the studies of Pohorecky and Wurtman (1971) which have established that in the adrenal medulla, the maintenance of the mammalian PNMT enzyme depends on the delivery of a high concentration of steroids via the corticomedullary portal system. Their evidence is summarised in two reviews (Pharmacol. Rev. 23 and 24, 1971 and 1972) and I shall now mention the salient features.

1. Most of the studies have been done on rats.

2. Hypophysectomy results in a rapid fall in medullary PNMT activity (half life = 3-5 days)

3. This activity is restored by corticotrophin administration but not glucocorticoid maintenance therapy.

4. Enzyme activity only begins to be restored by the administration of amounts of glucorticoids which give peripheral arterial concentrations fifty to one hundred times normal.

5. Thus the high concentrations of glucocorticoids found in the adrenal medulla (one hundred to two hundred times peripheral levels) appear to be necessary for the maintenance of PNMT and the effect is thought to be related to the synthesis of the enzyme protein.

6. Other classes of steroid hormones do not appear to have a physiological action on PNMT activity.

However, it is interesting to note that they base little of their argument on the actual catecholamine content of these glands.

This is, no doubt, because they found that the changes in catecholamine content lag behind the changes in enzyme activity. After hypophysectomy, adrenaline content does decline but with a half life of 30 days (although it may be as high as 80 days) while noradrenaline does show an increase but not in proportion to the fall in adrenaline.

However, radioactive tracer studies have shown that the half life of adrenaline in intact rat's adrenal medulla is 7-14 days (Udenfriend and Wyngarden, 1956).

The discrepancy between the half life of adrenaline in intact and hypophysectomised animals can be explained by postulating that adrenaline synthesis still continues at a near-normal rate in spite of the very low PMNT levels found after one to two weeks. However, a much simpler explanation is that release of adrenaline is considerably reduced.

Effects of Corticotrophin Administration

In 1933 Houssay, Biasotti, Mayzocco and Sammartino found that the catecholamine content of dog adrenal glands fell to seventy per cent of the control value after two days' treatment with anterior pituitary extract. However, after seven days' treatment the level had returned to normal. Seventeen years later, Houssay found a fifty percent reduction in the catecholamine content of adrenal glands of toads hypophysectomised three to five weeks previously (Houssay, Gerschman and Rapela, 1950). They also found that the percentage of noradrenaline tended to rise from a mean of 45% in the controls to 53% in the hypophysectomised animals.

About this time Hökfelt (1951) was investigating the effect of corticotrophin and/or hypophysectomy on the noradrenaline and adrenaline content of rat adrenal glands:-

(a) After hypophysectomy the noradrenaline content doubled in two days and it stayed at this level for over ten days while, apart from an initial fall, adrenaline remained at its control level.

(b) A single injection of corticotrophin given to rats hypophysectomised two days previously was followed by a progressive fall in noradrenaline content to ten percent of the hypophysectomised control value after three hours. Over the same period adrenaline content rose to about twice its initial value.

(c) Corticotrophin was given to intact and hypophysectomised rats three times daily for ten days. The intact rats showed depletion of adrenaline to two thirds of its control value while noradrenaline was not significantly affected. The adrenaline content of the

liver increased which presumably indicates adrenal release. The hypophysectomised rats were restored to a state similar to intact untreated rats.

Eränkö (1952) also used a biochemical method to assay the catecholamine content of rat glands exposed to five to ten days treatment with corticotrophin. He confirmed that there was no significant alteration in noradrenaline but adrenaline content was again observed to fall.

Another interesting observation is that of Harrison and MacKinnon who were investigating the palmar anhidrosis which was seen in response to intravenous adrenaline. Corticotrophin administration mimicked the anhidrosis while cortisol itself did not have any effect. Furthermore, the responses to stress and to adrenaline are prevented by phenoxybenzamine. (See Harrison and MacKinnon 1966).

Luft and Von Euler (1952) are often quoted for having shown that corticotrophin and cortisone administration reduces noradrenaline excretion in man. This work is based on one patient and their graph does show a fall in noradrenaline, with no change in adrenaline excretion, during the period of corticotrophin administration. However, with cortisone, the claimed fall in noradrenaline excretion is the continuation of a trend which started well before the period of administration of the latter drug.

However, a considerable amount of histological work has been done on the influence of corticotrophin or its lack on the adrenal cortex. Thus it is unlikely that any marked morphological changes in the medulla resulting from these procedures, have gone unnoticed.

The work that really gave us an incentive to investigate the influence of corticotrophin on adrenal medullary secretion was that of Wurtman, Casper, Pohorecky and Bartler (1968). There are several pieces of evidence in this paper which support the idea that the anterior pituitary-adrenocortical axis modulates catecholamine secretion. However, this does not appear to have occurred to the authors who interpret all their results in terms of the action of the anterior pituitary-adrenocortical axis on methylation and adrenaline output.

They used a reliable fluorimetric technique to assay the adrenal noradrenaline and adrenaline output in response to insulin hypoglycaemia. Intact dogs were compared with animals hypophysectomised three to five months previously. Some of the latter animals had received corticotrophin (40 units/day) during the previous month.

The dose of insulin was adjusted so that the extent to which the blood glucose level fell did not differ significantly between the three groups of animals. The authors seem to be trying to disguise the point I want to make by quoting the peak response as adrenaline output and adrenaline percentage of the total output. However, from their figures I have calculated peak total catecholamine output in response to hypoglycaemia in the three groups.

	Resting Output			Peak Response			A%
	NA	A	Tot	NA	A	Tot	
Normal	1.6	7.5 ± 0.01	9.1	7.0	31.9 ± 6.1	38.9	82.0 ± 4.2
Hypophysectomised	2.6	4.2 ± 0.7	6.8	9.5	15.6 ± 4.9	25.1	62.2 ± 8.3
Hypophysectomised & Corticotrophin	3.1	7.2 ± 2.9	10.3	32.3	74.4 ± 24.5	107	69.7 ± 3.5

Apart from its effect on methylation, hypophysectomy reduces both the resting output of total catecholamine and the response to hypoglycaemia. The corticotrophin more than restored the resting output and considerably potentiated (by $2\frac{1}{2}$ - 3 fold) the peak response to hypoglycaemia.

The increased resting output and potentiated insulin induced response cannot be entirely due to increased PNMT activity as the noradrenaline output is also increased (- by over three fold in the peak response).

PNMT activity

Normal	123.1 ± 10.4	mμM/gland
Hypophysectomised	28.8 ± 3.5	mμM/gland
Hypophysectomised & Corticotrophin	190.0 ± 24.9	mμM/gland

Their PNMT activity results are also worth considering. It is strange that the activity in the corticotrophin treated group should be higher than the intact animals when the former animals have a lower adrenaline percent in the output. (A suggestion of ours is that the secretion of catecholamines induces PNMT. c.f. the effect of neural stimulation). Furthermore, after three to five months, a fall in activity to a quarter of that in the intact animal is only associated with a reduction from 82% to 62% in the proportion of adrenaline secreted.

When they assayed the catecholamine content of the glands (μg/gland) they found that there was no difference between the noradrenaline contents (c. 200 μg/gland) but the adrenaline contents were very different.

	Adrenaline content of glands	
Intact	520 ± 100	µg/gland
Hypophysectomised	260 ± 60	µg/gland
Hypophysectomised & Corticotrophin	1260 ± 60	µg/gland

These adrenaline figures do not correlate well with the PNMT activities and neither is the absence of change in noradrenaline content what one would expect from their theory.

There is one further piece of supporting evidence for the hypothesis that the anterior pituitary-adrenocortical axis participates in the control of catecholamine release. They publish three graphs (one typical of each group) showing noradrenaline and adrenaline output against time. The potentiation of noradrenaline and adrenaline output by corticotrophin is very clear but in both the hypophysectomised animals (i.e. the corticotrophin treated one as well) catecholamine output rises sharply to a peak at about fifty minutes and then falls, although there is no evidence that the blood glucose has begun to rise. However, in the intact animal, catecholamine output rapidly rises to near its peak, within twenty minutes and stays elevated (still gradually rising) for ninety minutes after the insulin injection.

The differences between the time course of these two responses cannot be explained by the blood glucose levels which all fall to the same level and show no tendency to rise again. However, insulin hypoglycaemia is known to release corticotrophin from the anterior pituitary and this could be participating in maintaining the catecholamine release seen in the intact but not, of course, in either of the hypophysectomised animals. N.B. The endogenously released corticotrophin would not necessarily potentiate the release to the same extent as the large dose administered daily.

Goldfien, Sheref Zileli, Despointes and Bethune (1958) measured both canine adrenal catecholamine and 17 - hydroxycorticosteroid output during insulin hypoglycaemia. These results could have been very interesting but they are, unfortunately, too scattered and inconsistent for any useful conclusions to be drawn.

REVIEW OF TECHNIQUES AVAILABLE FOR CATECHOLAMINE ASSAY

There are four basic types of method which have been used for the determination of catecholamines:-

1. Biological assays

   See reviews of Gaddum (1959) and Callingham (1967)

2. Chemical techniques

   These are dependent on the formation of fluorescent derivatives (see reviews of Udenfriend (1958, 1962) and Callingham (1967)

3. Isotope derivative techniques

   These depend on enzyme specificity and the formation of radioactively labelled derivatives (see Engelman and Portnoy (1970)).

4. Radioimmunoassay

   Not yet developed.

The techniques differ in their sensitivities and whether they measure total catecholamine (by this term I mean adrenaline plus noradrenaline) or if they differentiate between adrenaline and noradrenaline. Other important considerations are reliability, the number of samples handled in one batch and cost.

After a survey of the literature and discussion with authorities in the field of catecholamine assay, we decided that a chemical technique using spectrophotofluorimetry would be most suitable for our adrenal effluent assay.

We developed an adequate assay for the fundamental part of our research and, once familiar with it, examined the modifications necessary

to assay catecholamines in peripheral plasma and urine.

Before discussing our assay in detail, I shall briefly discuss the three other approaches to catecholamine assay.

1. Bioassay

Catecholamines were first detected by their pharmacological properties and these led to biological methods of quantitative assay.

Although bioassay has been largely replaced by the various biochemical techniques available, when efficiently and carefully exploited it can be several orders more sensitive than the latter techniques, and does not require expensive apparatus - (it is commonly used in the developing countries).

Concerning the assay of catecholamines released from the adrenal medulla, there are three approaches:-

(a) Use of the experimental animal's own tissues to detect changes in circulating catecholamine levels. The tissues (e.g. nictitating membrane, perfused hind limb) are usually denervated prior to the experiment which both abolishes neuronally mediated effects and leads to the development of denervation supersensitivity. Reuptake blockers such as cocaine have also been used to increase sensitivity.

(b) Isolated organ assays

Samples removed from animals (e.g. taken from adrenal vein) and assayed on standard isolated tissue preparations (e.g. cat blood pressure and chicken caecum - see Gaddum 1959). These techniques usually involve an extraction procedure which makes them more specific and can be extremely sensitive. Harvey and Pennefather (1962) have modified the rat uterus inhibition technique of Gaddum and Lembeck (1949) by using

electrical stimulation to produce the contractions and claim to be able to detect picogram qualities of adrenaline.

Much of the pioneer work on the urinary excretion of catecholamines under different conditions, was done with isolated organ bioassay and it has been applied with varying degrees of success to peripheral plasma estimations.

(c) Superfusion techniques

Bioassay has recently returned to fashion with the exploitation of the superfusion technique by Professor J.R. Vane's group. It involves a cascade of isolated tissue preparations down which the experimental animals's own blood passes in an extracorporeal circuit (Vane 1966).

This technique combines the degree of sensitivity and specificity which can be obtained by careful selection of tissue preparations with the great advantage of "on line" assay (i.e. the assay result is obtained during the animal experiment and not days later as happens when a batch of samples is collected).

The main criticisms of this method are that other biologically active substances in the blood could interfere with the isolated tissue preparations and that it only detects changes in the level of circulating hormones.

However, we did consider using a superfusion cascade for our differential estimation of the catecholamines in adrenal venous blood as it would have had the following advantages:-

(i) On line assay

(ii) The adrenal blood would have been fed into a flow of artificial perfusate upstream of a Watson-Marlow pump supplying the cascade of

isolated organs. Catecholamine secretion would have been determined with respect to time and would have been independent of changes in blood flow through the gland.

(iii) Due to the very high concentrations of catecholamines in adrenal venous blood we could have used a dilution of the adrenal outflow and probably been able to disregard any other substances in the blood.

(iv) Peripheral venous blood would have been alternated with adrenal effluent so as to provide a reference base line for our isolated tissues.

However, the cascade should be set up in parallel with the whole animal preparation and we decided that we did not have the man-power or financial resources to attempt this approach.

2. Chemical techniques based on fluorescent derivatives - see later.

3. Isotope derivative techniques (Engleman, Portnoy and Lovenberg 1968; Engelman and Portnoy, 1970).

This method depends on the specificity of catechol-O-methyl transferose for noradrenaline and adrenaline. It converts them to normetanephrine and metanephrine by the addition of a ^{14}C-methyl group using S-adenosyl-L-methionine-methyl-^{14}C as the donor. It is of course imperative to completely separate the labelled donor from the newly formed metanephrines and to accomplish this, a rather involved extraction procedure is used. The metanephrines are absorbed on to a carboxylic acid ion exchange resin, eluted off and converted to vanillin. This is extracted into toluene for scintillation counting. The number of steps involved and especially the enzymic conversion leads to low and irregular recoveries. However, the disadvantage of this is neatly circumvented by

the addition of tracer amounts of tritiated noradrenaline and adrenaline to the plasma samples. The scintillation counter separately counts ^{14}C and ^{3}H giving each sample an internal recovery factor.

The enzymatic double isotope derivative assay is increasingly used for peripheral plasma total catecholamine assays and especially in clinical departments which did not previously attempt them. This is probably because the technical departments detailed to do the assays are more likely to have access to a scintillation counter than an adequately sensitive fluorimeter and furthermore, are unlikely to have experience with the exacting fluorimetric techniques required for success.

The modification of this technique described for differential assay of noradrenaline and adrenaline is in my opinion far less satisfactory as it depends on thin layer chromatography to separate the two catecholamines before enzymatic conversion. It is difficult for even the most conscientious workers to achieve routinely complete separation of the two substances by this technique (Personal communications from Dr. C.M.Yates and Dr. T.B.B. Crawford). The users of the assay claim to add traces of both noradrenaline and adrenaline but this will give no internal control over the differential aspect of the assay as both are tritium labelled and thus indistinguishable.

My final comment is that a scintillation counter has the "advantage" of producing a neatly typed-out set of numbers and thus a "result" can always be claimed. (This applies particularly to the differential assay technique).

4. Radioimmunoassay

The development of an assay with a similar degree of sensitivity to those currently available for steroid hormones would greatly benefit catecholamine research in general and completely transform peripheral plasma studies.

So far attempts to raise antibodies to unmodified catecholamines have failed but research is in progress (Dr.W.M. Hunter's unit *) to find a suitable antigenic derivative. Once this is achieved then a reliable method of producing this derivative in catecholamine-containing samples from various sources, will have to be established.

* M.R.C. Radioimmunoassay Team,
Forrest Road Laboratories,
Edinburgh.

METHODS

METHODS - WHOLE ANIMAL EXPERIMENTS

A - SELECTIVE CATECHOLAMINE RELEASE

Objective

Our objective was to find out if a discrete reflex stimulus would selectively release one or other adrenal catecholamine in either the dog or the cat. In each species, we investigated whether the ratio of noradrenaline to adrenaline in the increment released by the baroreceptor reflex was significantly different from that released during chemoreceptor stimulation.

Outline of Technique

The effect of the baroreceptor and chemoreceptor reflexes on noradrenaline and adrenaline secretion from the adrenal medulla was investigated in:-

(a) Dogs anaesthetised with chloralose-urethane

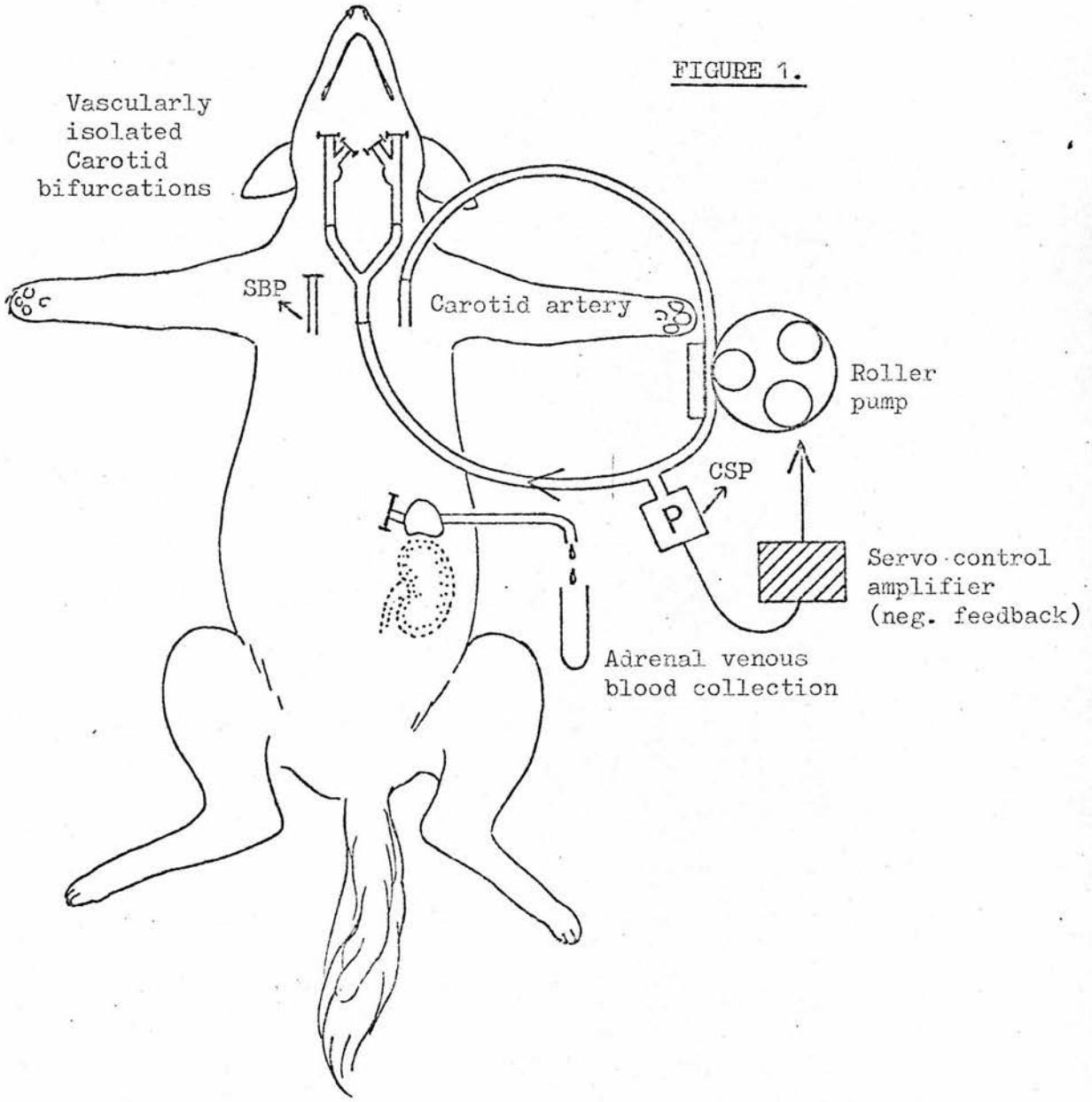
(b) Cats anaesthetised with sodium pentobarbitone

The carotid bifurcations were vascularly isolated and perfused by a roller pump at constant pressure with the animal's own arterial blood. A servo amplifier system controlled the speed of the pump and enabled us to set the carotid sinus pressure at will. (See figure 1). p 84

Baroreceptor tests were performed by lowering the carotid sinus pressure. Chemoreceptor tests were performed by lowering the oxygen tension of the blood perfusing the carotid bifurcations either by changing from arterial to venous blood or by infusing the reducing agent, sodium dithionite, which combines with the oxygen in the blood. The duration of the periods of reflex stimulation was one minute in the dog and two minutes in the cat.

The venous effluent from the left adrenal gland was collected and the catecholamine extracted before being assayed for noradrenaline and adrenaline by the trihydroxyindole spectrophotofluorimetric technique.

FIGURE 1.



CAROTID BIFURCATION PERFUSION AND ADRENAL VENOUS BLOOD COLLECTION

Surgical Protocol

1. Routine initial surgery performed (i.e. tracheal cannulation and hind or forelimb vein cannulated for anaesthetic infusion).
2. Carotid bifurcations prepared for vascular isolation and perfusion. p88
3. Vagi cut in the neck (We usually waited half an hour between sectioning the two nerves).
4. Venous outflow of the left adrenal gland prepared for collection of blood. The animal was put on artificial ventilation once the abdomen was opened. p94
5. Half an hour allowed for haemostasis
6. Heparin administered intravenously (500 i.u./kg.)
7. Carotid perfusion system set up and carotid bifurcations vascularly isolated
8. Blood gases and acid-base balance adjusted and anaesthetic state checked.
9. Collection of adrenal venous blood commenced. The waste blood not required for catecholamine estimation was collected and periodically re-injected into the animal
10. Alternate baroreceptor and chemoreceptor tests performed. p97

N.B. Details (e.g. manufacturer) of the instruments, reagents and drugs mentioned in this section on Methods are listed in the Appendix. p35

Anaesthesia

Dogs

Premedication - morphine sulphate (1 mg/kg) dissolved in 0.9% saline given sub-cutaneously half an hour before inducing anaesthesia.

Induction - A mixture of α-chloralose (55 mg/kg) and urethane (ethyl carbamate) (550 mg/kg) given intravenously.

Maintenance - Constant intravenous infusion of one tenth of the induction dose per hour - with due regard to the state of the animal's reflexes. (See p.106 for explanation of our choice of anaesthesia).

The chloralose-urethane mixture was given as a solution in equal volumes of 0.9% saline and polyethylene glycol resulting in a final concentration of 2.5% W/V α-chloralose and 25% W/V urethane. The use of polyethylene glycol enabled us to dissolve the relatively insoluble anaesthetic mixture in a manageable volume of fluid. There is no evidence from previous work (e.g. that of Ungar and co-workers) that the latter reagent has any adverse effects.

Cats

No premedication was used because the anaesthetic was given by the intraperitoneal route.

Induction - Sodium pentobarbitone "Nembutal" given intraperitoneally in a dose of 30 mg/kg.

Maintenance - Constant intravenous infusion of one tenth of the induction dose per hour (volume increased with saline) with due regard to the state of the animal's reflexes.

(See p.106 for explanation of our choice of anaesthesia).

Respiration, Blood Gases and Acid-Base Balance

Once the abdomen had been opened, the animals were put on positive pressure ventilation with a Starling "Ideal" pump using either air or a gas mixture enriched with oxygen (25-100%).

The respiratory rate was set at 25 per minute for both dogs and cats.

The stroke volume was adjusted with reference to the arterial blood gas tensions and ventilatory efforts of the animal. A sample of arterial blood was taken every ten to twenty minutes and the PaO_2, $PaCO_2$, and pH measured using a Radiometer Blood Gas Microanalysis system.

The $PaCO_2$ and pH were kept at the normal levels for the two species and any base deficit corrected by giving molar sodium bicarbonate according to the Singer-Hastings nomogram (1948).

The PaO_2 was usually kept well above normal so as to minimise any chemoreceptor component during the baroreceptor tests. However, it had to be lowered when we wanted to use venous blood (without sodium dithionite infusion) as a hypoxic chemoreceptor stimulant.

Temperature

A rectal probe and thermostat maintained the animal's body temperature at 37°C with the aid of a heating pad (i.e. small electric blanket under the animal) and infra red lamps.

Blood Loss

Systemic arterial blood pressure was monitored throughout the experiment and maintained by the intravenous administration of Dextran (prewarmed to 37°C in a waterbath). The solution usually contained glucose but, during the period of adrenal venous blood collection, we used dextran solution without glucose - (Changes in blood glucose are known to alter adrenal catecholamine output - see Introduction). p 38

Bladder Reflexes

In our preparations the bladder was drained by catheter or suprapubic puncture as there is evidence that the stimulus of a full bladder can modify activity in the central nervous system. (eg.Taylor 1968)

CAROTID BIFURCATION PERFUSION

A roller pump (Watson-Marlow MHRE) perfused both vascularly isolated carotid bifurcations at constant pressure with blood taken centrally from a common carotid artery. The carotid bifurcations were vascularly isolated so as to prevent the hypoxic blood reaching the brain and having a central action during chemoreceptor stimulation. Also it was easier for the pump to deliver the flow required to maintain any desired pressure. However, the flow through the system had to be sufficient to allow an efficient change from oxygenated to hypoxic blood.

For chemoreceptor stimulation, a Y-piece (not shown on the diagram) enabled either the carotid arterial supply to be switched to venous blood (obtained centrally from external jugular vein) or the infusion of the reducing agent, sodium dithionite.

A pressure transducer sited down stream of the pump enabled our servo-system (set on negative feedback) to hold carotid sinus pressure at any desired level by controlling the speed of the pump. Thus we could dial any required pressure and performed baroreceptor tests by lowering the perfusion pressure. There was a gradient down the tubing from the transducer side arm to the carotid sinus and we ensured that the cannulae into the carotid bifurcations were of the same length so that the carotid sinus pressures were equal on the two sides.

Surgery

Sufficient lengths of both common and carotid arteries were exposed for two-way cannulation.

The carotid bifurcations were prepared for isolation by placing ligatures around the:-

External carotid arteries - cranial to the origin of the lingual artery.

Internal carotid arteries

Occipital arteries

Ascending pharyngeal arteries

The superior thyroid arteries were tied off immediately and the vagi prepared for section. Great care was taken to keep the dissection as far away as possible from the carotid body and the sinus nerve. (The venous drainage of the carotid body is said to be particularly vulnerable to damage).

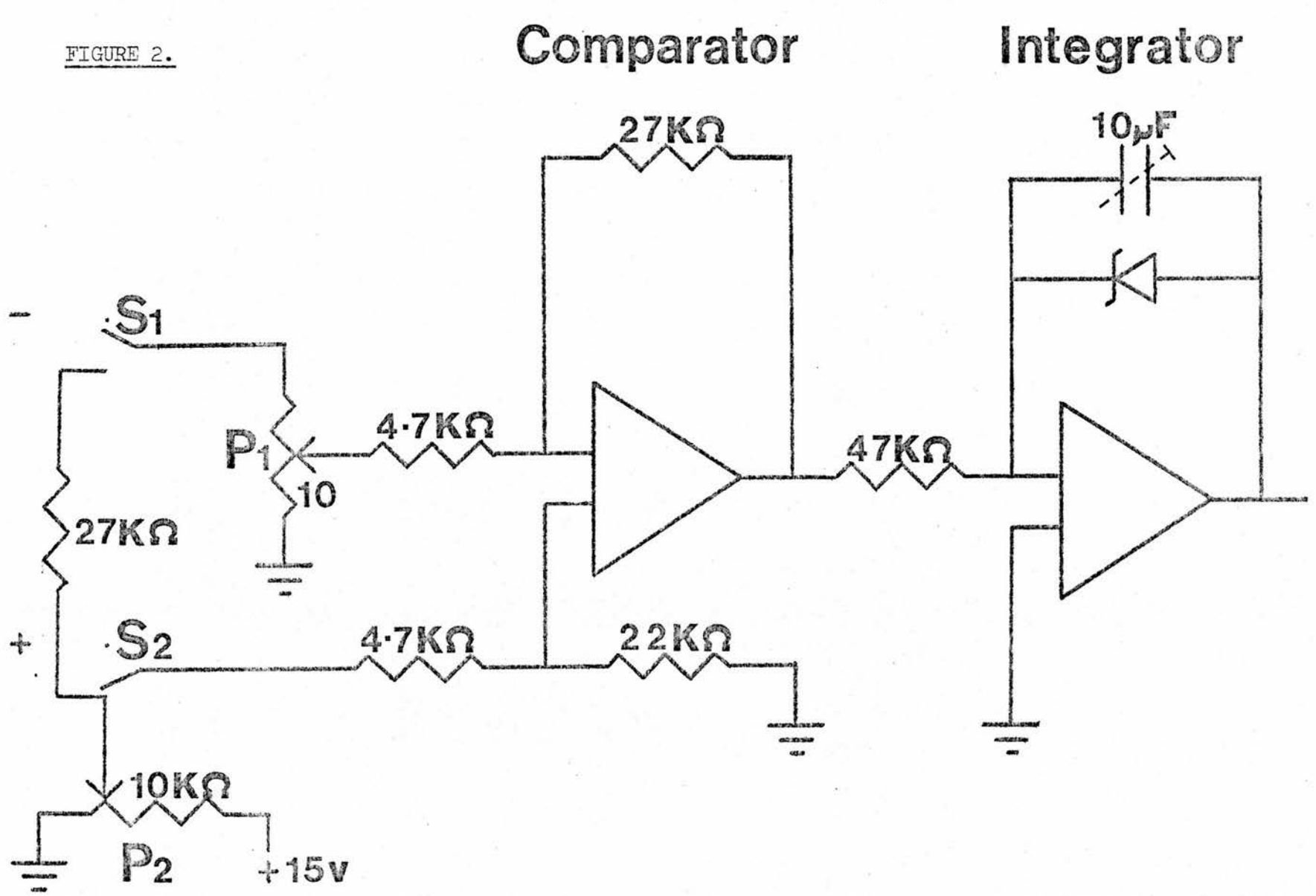
The lingual arteries were left open so as to ensure an adequate flow of blood through the carotid bifurcations. We assumed that the anastomosis between the lingual artery and the brain is negligible.

Once the perfusion apparatus had been set up and adjusted, we tied off the ligatures in order to vascularly isolate the carotid bifurcations. The roller pump slowed due to the reduced requirement for flow.

Servo-control system

The details can be seen in the circuit diagram (Figure 2) but, ps in brief, the servo-system is based on two operational amplifiers. The first is the comparator into which is fed the voltage from the pressure transducer and also that from an adjustable (clock) potentiometer. The difference between these two voltages is amplified and fed into the second amplifier, the integrator, which controls the speed of the Watson-Marlow pumps. (One virtue of these pumps is that their speed is determined by

FIGURE 2.



CIRCUIT DIAGRAM FOR SERVO CONTROL AMPLIFIER SYSTEM

a potentiometer which can be bypassed and substituted with another voltage input). Any change in output from the comparator is integrated and added to or subtracted from the voltage held by the second amplifier which drives the pump. The operational amplifiers have a positive and negative input and hence the output from the comparator is the difference between the pressure transducer and clock potentiometer voltages. The polarity of any such difference and therefore, that of any change in the comparator's output depends on the orientation of the two input voltages. (See circuit diagram). p90

Thus the servo-system can be set for either positive or negative feedback and in these modes it can:-

(a) Maintain a pressure set by the clock potentiometer

(b) Follow an external pressure when the clock potentiometer is bypassed and replaced by an input from a second pressure transducer.

Examples of the four basic modes of action of the servo-system are:-

1. Pressure set by clock potentiometer
   (a) Negative feedback - Carotid bifurcation perfusion at constant pressure.
   (b) Positive feedback - Venous Capacitance technique at constant pressure (See Appendix IV). p28
2. Following an external pressure.
   (a) Negative feedback - Perfusing a region (e.g. hindlimb, carotid bifurcations) at a pressure equal to Systemic Blood Pressure.
   (b) Positive feedback - Maintenance of Portal Venous Pressure at Central Venous Pressure (See Appendix IV).

Baroreceptor tests

These are performed by changing the setting on the clock potentiometer and thus lowering carotid sinus pressure from, say, 140 mm Hg to 80 mm Hg (See Figure 3). p93

The animals were usually breathing an oxygen-rich mixture which gave a high arterial PO_2. Thus the chemoreceptor component in response to the fall in carotid body perfusion pressure should have been minimal. (see p.12)

Chemoreceptor tests

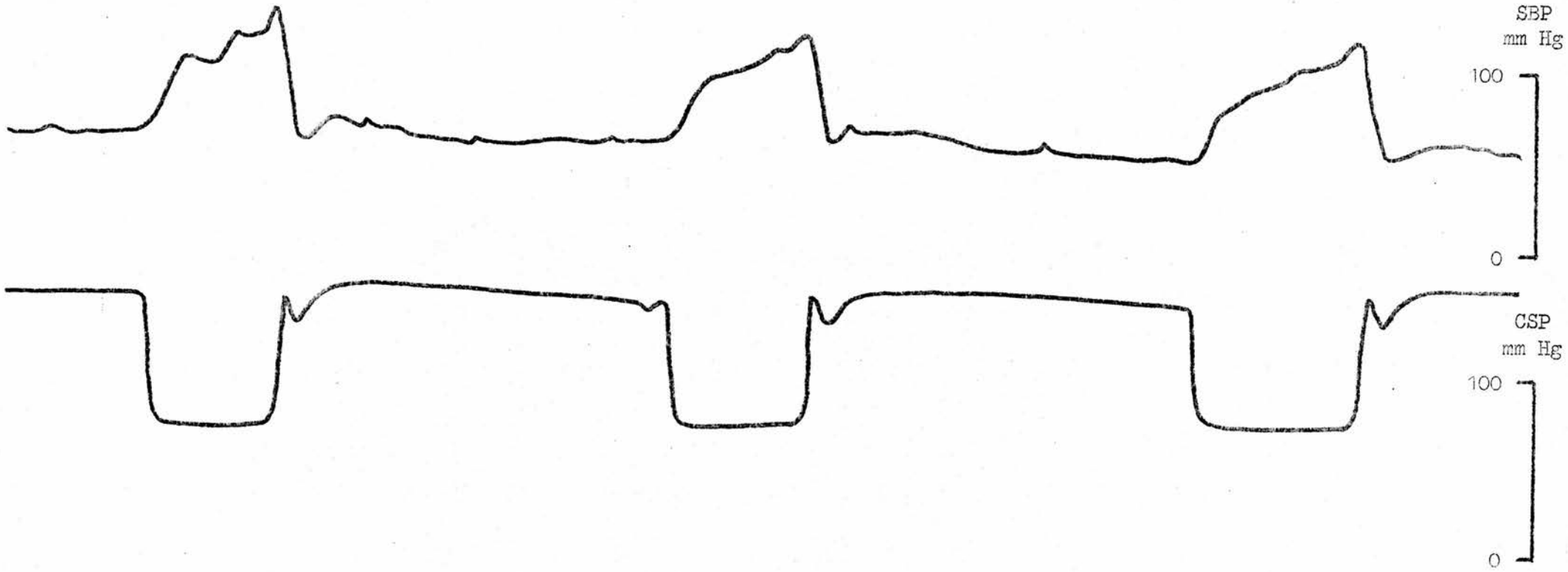
These were performed in one of two ways:-

1. Changing the carotid bifurcation perfusate from arterial to venous blood taken centrally from the external jugular vein - this was usually only a mild stimulus.

2. Infusing the reducing agent, sodium dithionite, into the arterial blood perfusing the carotid bifurcations. A 0.25 M solution of sodium dithionite was freshly prepared under liquid paraffin and infused into the carotid perfusate. By altering the rate of infusion, graded hypoxic stimuli could be given down to a PaO_2 of zero. A blood gas sample told us the degree of hypoxia, hypercapnia and acidaemia achieved with each infusion.
(The use of sodium dithionite is justified on p.109).

FIGURE 3.

An example of the reproducibility of
our baroreceptor tests - taken from DA1



ADRENAL VENOUS EFFLUENT COLLECTION

Dog

The adreno-lumbar vein normally collects its tributaries from the body wall, receives a small branch from the adrenal gland and then runs the short distance from the gland to join the renal vein or the inferior vena cava. It is usually impracticable to cannulate this latter short length of vein and so we used the well established retrograde cannulation technique.

A ligature is placed around the adrenal vein in the region between the gland and the large collecting vein (inferior vena cava or renal vein). The tributaries of the adreno-lumbar vein are tied off and a short length near the gland is prepared for cannulation towards the gland.

Soft and flexible polyethylene tubing was used for the cannulation and was as wide as possible in order to reduce resistance.

Cannulation was performed after heparinisation and setting up the carotid perfusion. The end of the cannula was clamped and the outflow to the great vein left untied until just before adrenal collection was commenced so as to minimise blood loss.

Great care was taken not to damage the gland or its nerve supply.

Cat

The venous drainage of the adrenal gland in the cat is similar to that in the dog, although it appeared to be more variable in the former species.

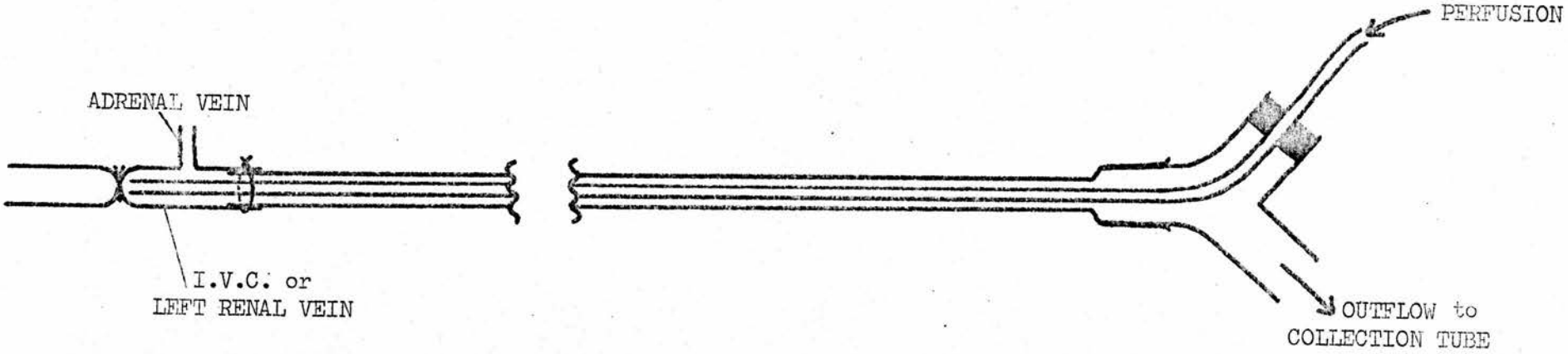
We often found that the adreno-lumbar vein was so small that we could not cannulate it with the narrowest cannula which we considered reasonable to use. The resistance of a very narrow cannula would have led to an abnormal back pressure in the gland. (There is evidence that this can increase catecholamine discharge. Kayaalp and McIsaac, 1968).

Other workers in the past have used the vena caval or, where necessary, renal vein pocket technique. This involved ligating the collecting vein above and below the entrance of the adrenal vein and creating a pocket from which the adrenal venous effluent was sampled. However, if one ties the adreno-lumbar vein to reduce haemorrhage, which one must if the preparation is to last for any length of time, the dead space of the system becomes relatively large. Thus this technique is only satisfactory for samples collected over large periods of time (e.g. 15 minutes) and would have been unreliable in our situation where stimuli lasted only 2 minutes. We overcame this disadvantage by the following technique:-

A double lumen cannula was made using a "Portex" Y-piece and two cannulae of different diameters. (Figure 4). Sucrose solution (10%) was perfused down the inner cannula at a flow of 4 mls/minute and continuously washed the blood out of the pocket. The washing action was made more efficient by positioning the cannula so that the outflow from the gland was situated between the ends of the inner and outer cannulae (see diagram). p9

We checked (by using a finger stall to imitate the caval pocket) that, at the above flow rate, the pressure gradient along the outer lumen was less than 10 mm Hg. N.B. Sucrose solution was used because it did not alter the ionic content of the sample (see section on catecholamine extraction) and increased the sedimentation rate of the red cells (thus making centrifugation quicker). p13

FIGURE 4.



ADRENAL VENOUS BLOOD COLLECTION SYSTEM

used for CATS

PROTOCOL FOR REFLEX TESTS

Baroreceptor and Chemoreceptor tests were performed alternately at ten or twenty minute intervals.

The duration of the periods of reflex stimulation and adrenal venous blood collection in each species were:-

Dogs - One minute
Cats - Two minutes

Blood Gas Tension Sampling - Samples of the blood perfusing the carotid bifurcations were taken from a three-way tap sited upstream of the Watson-Marlow pump so that sampling did not affect carotid sinus pressure. The following samples were taken:-

Baroreceptor test - Control sample taken just before the start of the control adrenal venous blood collection.

Chemoreceptor test - Control sample taken just before the start of the control adrenal venous blood collection.
Test sample taken just before the end of the period of hypoxic chemoreceptor stimulation.

Adrenal Venous Blood Collections for each test were as follows:

CONTROL - For the one/two minute period prior to the period of reflex adrenal stimulation.

TEST - Started half a minute after the commencement of the period of reflex stimulation in order to allow for the dead space of the collection system.
For the chemoreceptor tests we also allowed for the time (about ten seconds) taken for the hypoxic blood to pass through the carotid perfusion circuit.

We used larger stimulation and collection periods in the cats so as to obtain for the assay, quantities of catecholamine similar to those obtained in the dog.

Splanchnic Nerve Stimulation

This was only performed in dogs.

The greater splanchnic nerve to the adrenal gland was dissected out - (checked by observing the small rise in blood pressure when the nerve was electrically stimulated).

The nerve was then sectioned and the peripheral end placed on bipolar electrodes connected to a Grass stimulator. The nerve was kept moist under damp gauze.

Stimulation periods were of one minute duration and were performed at ten minute intervals (frequency = 10 per second; pulse duration = 2 msec voltage = 10 volts).

Adrenal venous blood collecting

For each test we took a control adrenal venous sample and started collecting the test sample thirty seconds after commencing stimulation.

Recordings

The following parameters were recorded during each experiment and are given in the Tables of Results. p29

Arterial Blood Gas Tensions - Samples were taken specifically for the baroreceptor and chemoreceptor tests (see protocol for reflex tests). p97

Systemic Blood Pressure - Taken from the femoral artery or common carotid artery not used for the carotid perfusion.

Carotid Sinus Pressure - Taken from the pressure transducer in the carotid perfusion circuit which also operated the servo control system.

Adrenal Blood Flow - The samples were collected in graduated 10 ml centrifuge tubes and the volume of each was recorded - (the volume of sucrose solution was subtracted).

Catecholamines - The noradrenaline and adrenaline contents of the adrenal venous blood samples were assayed and are given in ng/kg.min. in the Tables of Results.

The weight and sex of each animal was also recorded.

OUTLINE OF SELECTIVE RELEASE EXPERIMENTS

Introduction

I shall now outline the experiments we performed in dogs and cats to investigate specifically the possibility of selective adrenal catecholamine release. The details of the individual experiments are given in the tables of results. I have excluded experiments which were surgical failures or else deteriorated before we could perform any tests.

Dogs

DR1 - 5

Our standard surgical protocol was used (i.e. with carotid perfusion and adrenal blood collection). p85

Alternate baroreceptor and chemoreceptor tests performed.

Halfway through experiments DR1 - 3, guanethidine (2 mg/kg) was given in order to abolish the reflex increase in blood pressure which resulted from each test. (This was done to exclude the possibility that the increased adrenal blood flow during the reflex test was responsible for the increased catecholamine secretion).

DN 1 and 2

Our standard surgical protocol was used without setting up the carotid perfusion.

The greater splanchnic nerve was stimulated at ten minute intervals.

Near the end of experiment DN1 atropine (20 µg/kg) and later hexamethonium (2 mg/kg) were given. In DN2, the effects of the drugs were examined in the reverse order.

Cats

Cat 1-7

Our standard surgical protocol was used (N.B. the perfusion system for obtaining adrenal venous blood from cats). p95

Alternate baroreceptor and chemoreceptor tests performed.

At the end of experiment Cat 2 suberyl dicholine (100 µg/kg) was given into the carotid perfusion as a chemoreceptor stimulant.

In experiment Cat 4-7 hexamethonium (2-6 mg/kg) and later atropine (20-100 µg/kg) were given part way through the experiment. (N.B. Order of drug administration reversed in Cat 6 and 7).

B-ENDOCRINE MECHANISM OF ADRENAL MEDULLARY RELEASE

Objective

To investigate the influence of the anterior pituitary - adrenocortical axis on catecholamine secretion from the canine adrenal medulla with respect to:

1. Resting output
2. Neuronally mediated release evoked by the baroreceptor reflex.

Introduction

We required a stable preparation which would last for several hours and a method of delivering discrete and reproducible neural stimuli to the gland for we expected any humorally mediated adrenal medullary response to be of slower time course than the direct neural reflexes previously investigated. We also wanted to minimise any adrenocortical activity resulting from the generalised stress of our surgical and experimental procedures.

Our carotid sinus perfusion technique had the potential for delivering reproducible baroreceptor stimuli (See Figure 3) to the adrenal p9 medulla and we decided to employ it in our investigation of the influence of the adrenal cortex on catecholamine release from the medulla.

The following procedures were performed as described for dogs in the selective release experiments section:-

1. Anaesthesia - Chloralose-urethane after premedication with morphine sulphate. p85
2. Surgical protocol p85
3. Carotid bifurcation perfusion - p88

4. Chemoreceptor stimulation using venous blood (10 or 20 minutes duration). p92

5. Baroreceptor tests (1 minute duration) - five or ten minute cycles.

6. Adrenal venous effluent collection. p94

I shall now outline our two series of experiments. Further details of the individual experiments (e.g. timing, blood values) can be obtained from the tables of results. p29

First Series

In view of any background adrenocortical activity, we attempted to induce a maximal adrenocortical response by administering exogenous corticotrophin or provoking its release by means of prolonged carotid chemoreceptor stimulation. The preparation of corticotrophin used was Synacthen (Ciba).

1. Exogenous Cortictrophin

In experiments DA1-3 (with baroreceptor tests) and DN3 (denervated and with splanchnic nerve stimulation) we investigated the p98 action of a dose of 1 I.U./kg Synacthen.

Comment - from the work of Ganong (1963) we expected this dose to give a maximal adrenocortical response lasting for half an hour and Marotta (1972) had produced near maximal 11-OHCS secretion in dogs with a dose of 1.5 I.U./kg.

2. Endogenous Cortictrophin

DC1 - Influence of twenty minutes of carotid chemoreceptor stimulation on denervated left adrenal gland - (baroreceptor tests used to confirm denervation).

DC8 - Influence of 10 minutes of carotid chemoreceptor stimulation on innervated left adrenal gland which was subjected to baroreceptor tests at five minute intervals (the right adrenal gland had been denervated to prevent any central or other effects from the circulating catecholamines released from it by reflex adrenal stimulation).

Comment - Marotta (1972) produced a near maximal adrenocortical response in dogs with fifteen minutes of 10% oxygen breathing. This increase in corticosteroid secretion was greatly reduced by carotid sinus nerve section and completely abolished when both the carotid and aortic bodies were denervated. Anichkov, Malyghina, Poskalenko and Ryzhenhov (1960) had also previously shown that peripheral chemoreceptor stimulation activates the anterior pituitary-adrenocortical axis.

Second Series

We had two objectives when we planned this series of experiments. Firstly, to find out if the delayed release of catecholamine which we had observed after Synacthen administration and prolonged chemoreceptor stimulation was prevented by the administration of cycloheximide. Garren, Ney and Davis (1965) had shown in rats that cycloheximide blocked the release of corticosteroids in response to corticotrophin and we decided to use their dose (i.e. 50 mg/kg).

Secondly, we wanted to find the threshold of the hypoxia required during prolonged carotid chemoreceptor stimulation to produce an appreciable delayed-type catecholamine release. Thus, we used only moderate degrees of carotid hypoxia for the first prolonged chemoreceptor stimulus in each experiment.

DC2 - 7 had the following protocol:-

(a) Control collections of testing and baroreceptor test adrenal venous output (this base line determined over ten minutes).

(b) Moderate hypoxic carotid chemoreceptor stimulation (ten minutes duration).

(c) Collections of resting and baroreceptor (one or two) test adrenal venous output (thirty minutes).

(d) Cycloheximide - 50 mg/kg intravenously.

(e) Collections of resting and baroreceptor test adrenal venous output (twenty minutes)

(f) More severe hypoxic carotid chemoreceptor stimulation (ten minutes duration).

(g) Collections of resting and baroreceptor test adrenal venous output (twenty or thirty minutes).

(h) "Synacthen" 1 I.U./kg intravenously.

(i) Collections of resting and baroreceptor test adrenal venous output (twenty minutes).

SOME COMMENTS ON OUR WHOLE ANIMAL EXPERIMENTAL TECHNIQUES

A. CHOICE OF ANAESTHESIA

p85

By their very nature, all anaesthetics must cause some depression of the central nervous system and there was a case for using decerebrate animals. However, as this is a difficult operation to perform in dogs and any additional major surgical procedure would have increased our experimental failure rate, we decided to use anaesthesia for financial reasons.

The influence of various anaesthetic agents on cardiovascular reflexes has been shown by comparing the response to the relevant stimulus before and after administering the agent to a decerebrate animal. (See Heymans and Neil 1958). In dogs it is claimed by these authors that ordinary anaesthetic doses of chloralose and pentobarbitone do not unduly reduce reflex responses although the latter compound tends to produce more depression than does chloralose. However, there appears to be a species difference between the dog and the cat as shown by Neil, Redwood and Schweitzer (1949) who demonstrated that, in cats, chloralose selectively depresses the baroreceptor as compared to the chemoreceptor reflex. This phenomenon does not occur in the dog.

The early work of Elliot (1912) showed that various anaesthetic agents induced a depletion of catecholamine from the adrenal gland. Elmes and Jefferson (1942) found that in cats a pentobarbitone-morphine mixture gave the lowest rate of depletion (3% per hour) out of a number of anaesthetic regimes. Also with this species, Emmelin and

Stromblad (1952) found that chloralose gave a remarkably low rate of loss (0.1% per hour) compared with morphine-ether anaesthesia (11% per hour). This difference may be due in part to a combination of morphine's excitatory effect and the selective baroreceptor depression seen with chloralose in cats.

A further disadvantage of pentobarbitone is its hexamethonium-like action which is seen with high doses.(Exley 1959)

Thus for our work in dogs we decided to employ chloralose-urethane anaesthesia because it appears to have relatively little reflex depressant or adrenal depleting action and it is commonly used in studies of cardiovascular reflexes. The use of chloralose alone has the disadvantage that the animals tend to awake suddenly during an experiment and so urethane is added.

However, we could not use chloralose for our cat experiments because of its effect on the baroreceptor reflex and instead, we chose pentobarbitone. The latter drug's ganglion-blocking action was a cause for concern and we took great care not to over-anaesthetise the animals. However, if it had not been for the cost of cats, we would have done some experiments on decerebrate animals.

Morphine premedication in dogs

We had to inject a large volume of anaesthetic into the dogs and in order to make handling easier (and less traumatic), we premedicated the dogs with 1 mg/kg of morphine half an hour prior to inducing anaesthesia.

When given in large doses to cats, morphine has been shown to release adrenal catecholamines. Elliot (1912) detected significant

depletion after a dose of 25 mg as did Vogt (1954) using 30-60 mg/kg. Outschoorn (1952) observed no loss after a single dose of 20 mg/kg but repeated injections every hour did result in adrenal loss. This may in part be due to morphine's excitatory action in the cat which is not found in dogs or man.

However, in dogs our premedication dose of 1 mg/kg was less than a tenth of the doses used in cats to obtain depletion and as it was given four to six hours before our sample collection began, we thought that its effect on our results would be negligible.

B. CAROTID BIFURCATION PERFUSION SYSTEM p88

We established the servo-amplifier controlled carotid bifurcation perfusion system as a method of delivering discrete reproducible reflex stimuli to the central nervous system, (See Figure 3). p 93 It is a simple system and when being used solely for baroreceptor mediated stimuli, it involves only minor surgery (i.e. that surgery necessary to reduce the flow requirements of the system such as tying off the external carotid arteries).

C. ADRENAL VENOUS BLOOD COLLECTION TECHNIQUE p94

Our adrenal venous blood collection technique (i.e. the double lumen cannula) which we devised for cats enabled us to use much shorter collection periods than had been employed by other workers. (e.g. Ten minutes - Fuerstein and Gutmann, 1971). Furthermore, that error involved in collecting small blood samples which is related to size of the individual drops was reduced.

D. THE USE OF DITHIONITE IN CHEMORECEPTOR STIMULATION p92

Introduction

The most straightforward way of stimulating the chemoreceptors in our preparation, was to change the carotid bifurcation perfusion from arterial to venous blood taken (centrally) from the jugular vein.

However, when we were comparing the chemoreceptor and baroreceptor reflexes we needed a high arterial oxygen tension to avoid a chemoreceptor component during the baroreceptor tests. Thus the venous PO_2 was usually not low enough to evoke significant cardiovascular responses and we did not want to alter the arterial PO_2 for the chemoreceptor tests because of possible central effects. We therefore needed another method of stimulating the chemoreceptors and there were a number of possible approaches.

1. Use of a drug to stimulate the chemoreceptors. p13

   (a) Histotoxic drug such as potassium cyanide. This has the following disadvantages:-

      (i) Difficult to judge the dose required for a short discrete stimulus

      (ii) Tachyphylaxis and cumulation if drug used repeatedly

      (iii) Recirculation

      (iv) Central toxic actions

   (b) Nicotinic agonist such as suberyl dicholine. This drug is superior to cyanide as it is broken down by pseudocholinesterase but still has some disadvantages:-

(i) Its action may not be specific to the chemoreceptors

(ii) It would have been useless when we came to investigate the action of hexamethonium on the chemoreceptor and baroreceptor reflex as the ganglion blocker would abolish suberyl dicholine's stimulant effect on the chemoreceptors.

(iii) Responses to chemoreceptor stimulant drugs cannot be expressed in terms of the physiological stimulus; that is hypoxia.

2. Use of a donor animal to supply the blood perfusing the carotid bifurcations. This is the ideal method but was economically unfeasible.

3. Lower the PO_2 of the animal's own blood by:-

(a) Changing arterial PO_2 - but this may have central effects.

(b) Using a deoxygenator

M. Scott who has done a lot of work in this field, claims that they are easy to use, give very stable blood gas levels and frothing does not present a problem. She also considers that the release of vaso-active substances from the blood is of no consequence. However, such a system has a large extracorporeal volume which needs to be primed with blood from a donor animal or else the volume of plasma substitute required would cause considerable dilution of the blood.

(c) Electrically stimulate the nerves to a limb and thus lower the venous PO_2 (and raise the PCO_2) by working the muscles. We had never tried this method and considered that its establishment as a satisfactory technique would be difficult and costly in animals.

(d) Chemically lower the PO_2 of the arterial blood used to perfuse the carotid bifurcation by infusing a reducing agent. Sodium dithionite had been used for in-vitro studies of haemoglobin to reduce oxyhaemoglobin and methaemoglobin.

Sodium Dithionite

This chemical compound reacts rapidly with dissolved oxygen to yield sulphate and hydrogen ions.

It had the following advantages:-

(a) Graded stimuli could be applied to the chemoreceptors depending on the amount of dithionite infused into the blood and a blood gas sample would indicate the degree of the stimulus.

(b) The dithionite was acting through the physiological mechanism of hypoxia potentiated by acidaemia and also there was no evidence of tachyphlaxis.

(c) We did not have to change from arterial to venous blood and the animal could be continuously breathing an enriched oxygen mixture. As a consequence of the haemoglobin dissociation curve, it makes little difference to the amount of dithionite required to lower blood PO_2 to chemoreceptor stimulant levels whether the PO_2 is 100 or 400 torr.

(d) There is no recirculation problem as the small amount of sulphate which may build up in the blood appears to be harmless and it should be excreted by the kidneys.

I will later describe the experiments done to justify (1 - 4). p113

Chemistry

Sodium dithionite ($Na_2S_2O_4.2H_2O$) is a salt of dithionous acid (hypo/hydrosulphorous acid). The ion consists of two SO_2 groups joined by a long bond between the sulphur atoms.

It is used in the dye industry and also in Fieser's solution for removing oxygen from inert gases (Fieser's solution is aqueous sodium dithionite with 2-anthraquinonesulphonate as a catalyst).

Although it is reasonably stable in the solid form ($Na_2S_2O_4.2H_2O$) it decomposes in solution to form metabisulphite and thiosulphate.

$$2Na_2S_2O_4 \longrightarrow Na_2S_2O_5 + Na_2S_2O_3$$

Thus we kept measured amounts of the salt in sealed pots. Just before the chemoreceptor test we dissolved it in 10 mls of water and covered the solution with liquid paraffin.

Both metabisulphite and thiosulphate are oxidised in the presence of oxyhaemoglobin to sulphate.

The overall reaction for the oxidation of sodium dithionite is as follows, although the detailed chemistry of the higher oxy-acids of sulphur has not been fully elucidated.

$$2Na_2S_2O_4 + 3O_2 + 2H_2O \longrightarrow 4NaHSO_4$$

The formation of bisulphite increases the hydrogen ion concentration of the blood and further potentiates the hypoxic stimulus.

Experiments to validate the use of sodium dithionite

To justify the use of sodium dithionite as a chemoreceptor stimulant we had to confirm the following:-

"In vitro"

1. It combines in a quantitative manner with the oxygen in the blood

2. The reduction of oxyhaemoglobin is reversible

"In vivo" - when infused into the carotid bifurcation:-

3. The responses to chemoreceptor stimulation are dependent on the rate of infusion and the degree of hypoxia obtained.

4. The response to chemoreceptor stimulation is abolished by denervation of the carotid bodies.

In vitro experiments

100 mls of fresh blood in a beaker were covered with liquid paraffin and continuously agitated with a magnetic stirrer.

A Watson Marlow pump continuously sampled the blood (0.1 ml/min) and passed it through our Radiometer blood gas machine which gave a continuous record of PO_2 on a UV recorder.

We oxygenated the blood to a PO_2 of over 200 torr and then slowly infused sodium dithionite solution. The infusion of the reducing agent removed a constant amount of oxygen with respect to time and thus we drew a haemoglobin dissociation curve. This demonstrated the quantitative combination of dithionite with the oxygen in the blood.

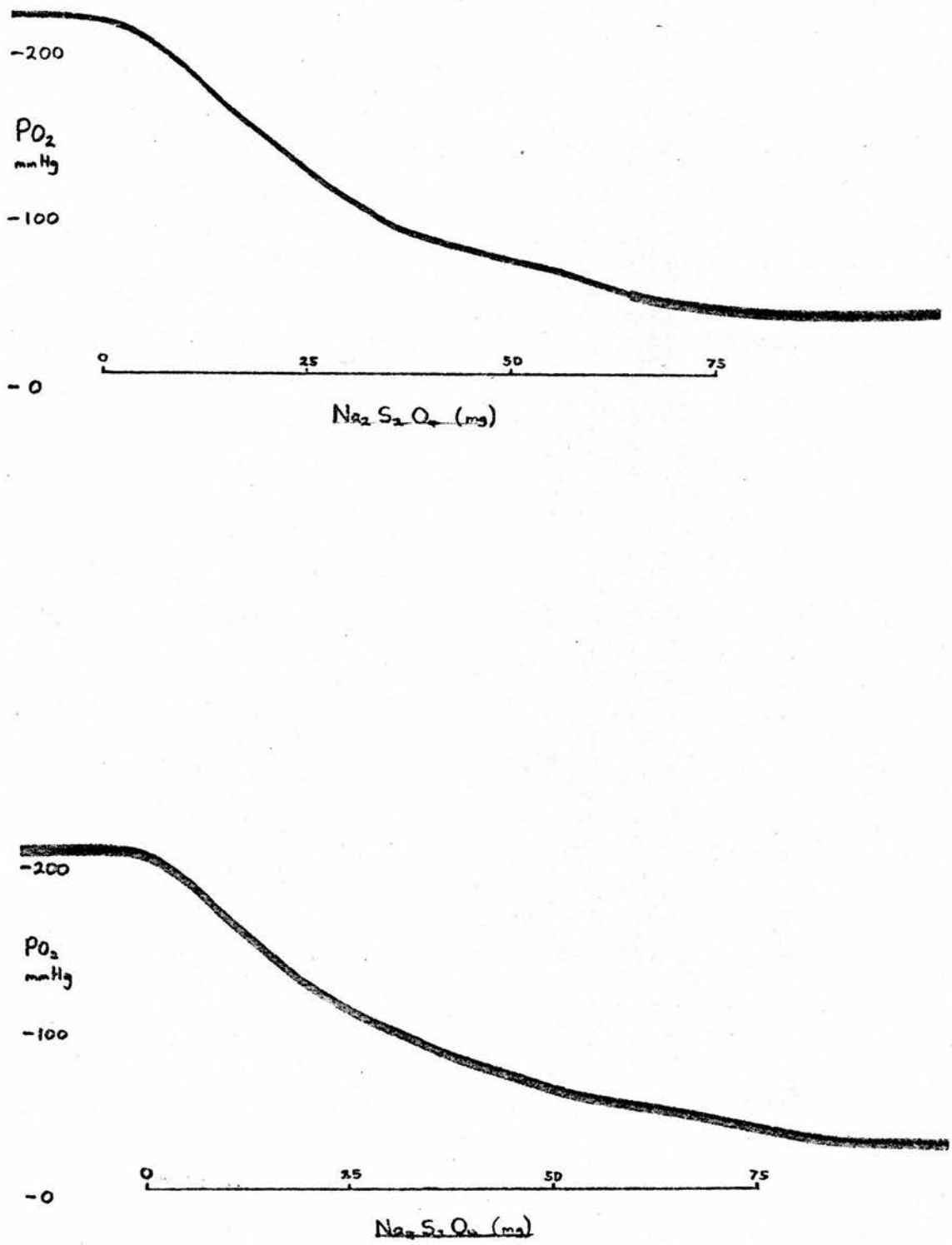
We then bubbled oxygen through the blood and returned it to a PO_2 of over 200 torr. A second infusion of dithionite at the same rate drew an identical curve and demonstrated the reversible nature of the reaction.

The above procedure was repeated in many samples and an example is illustrated (see Figure 5).

HAEMOGLOBIN DISSOCIATION CURVES OF WHOLE BLOOD PRODUCED BY INFUSION OF SODIUM DITHIONITE SOLUTION AT A CONSTANT RATE IN VITRO

(Blood reoxygenated between top and bottom curves)

FIGURE 5.



In vivo experiments

In two dogs we set up our carotid bifurcation perfusion system and recorded ventilatory responses with a pneumotachograph.

We examined the response to a series of one minute infusions of sodium dithionite into the carotid perfusate in either increasing or decreasing dosage.

One series of six different doses is illustrated:-

(a) Figure 6b shows the relationship between infusion rate of dithionite and degree of hypoxia achieved.

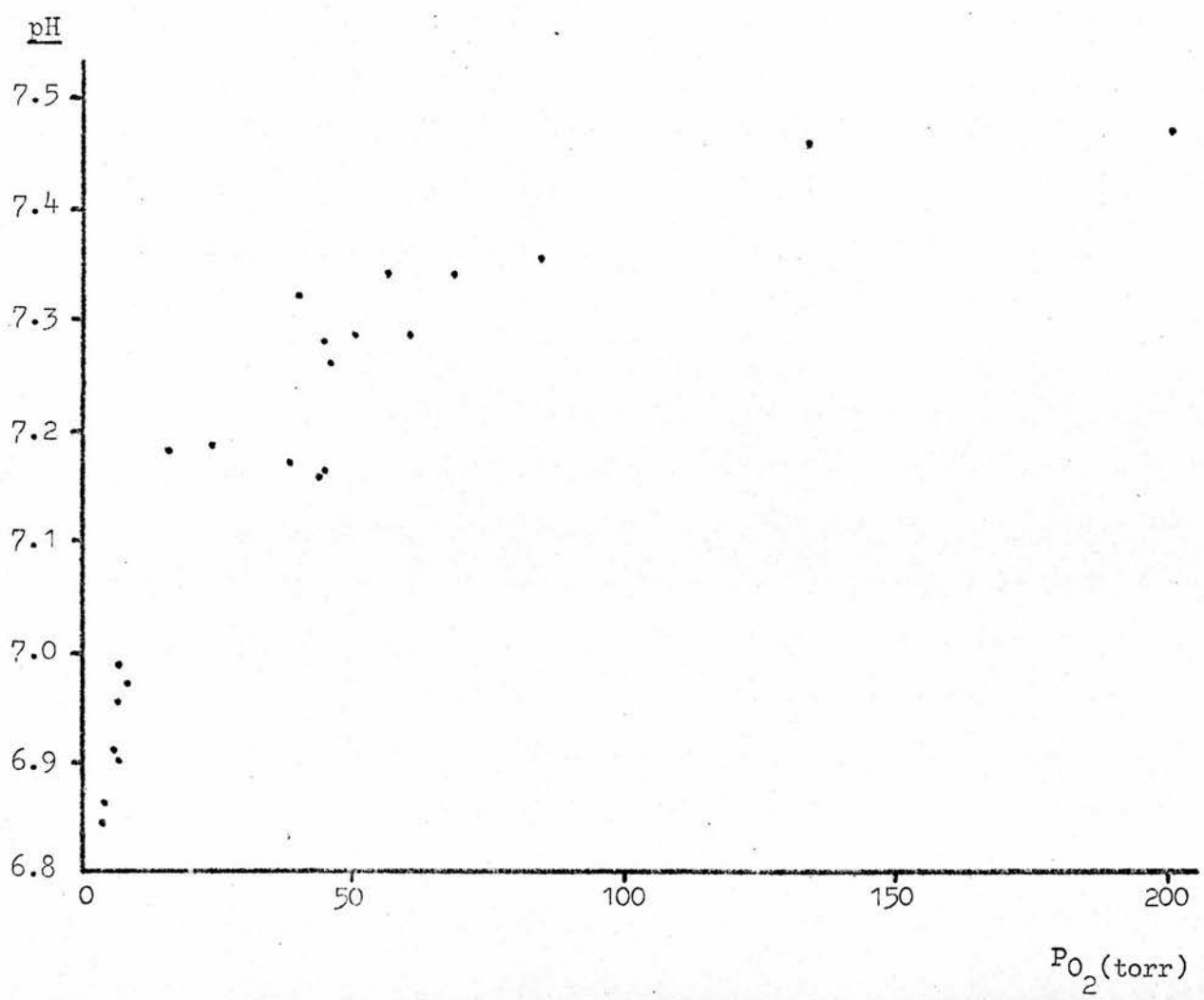
(b) Figure 7 shows the increase in ventilatory response with increasing stimulus

Using all the carotid perfusate blood samples I have plotted the relationship between pH and the PO_2 achieved with dithionite (Figure 6a).

Finally, we showed that the response to dithionite is abolished by injecting local anaesthetic (lignocaine 2%) into the regions of both carotid sinus nerves (see Figure 8).

FIGURE 6. a) THE RELATIONSHIP BETWEEN pH AND PO_2 IN CAROTID BLOOD REDUCED BY SODIUM DITHIONITE.

b) THE EFFECT ON PO_2 OF INCREASING RATES OF DITHIONITE INFUSION INTO CAROTID PERFUSATE



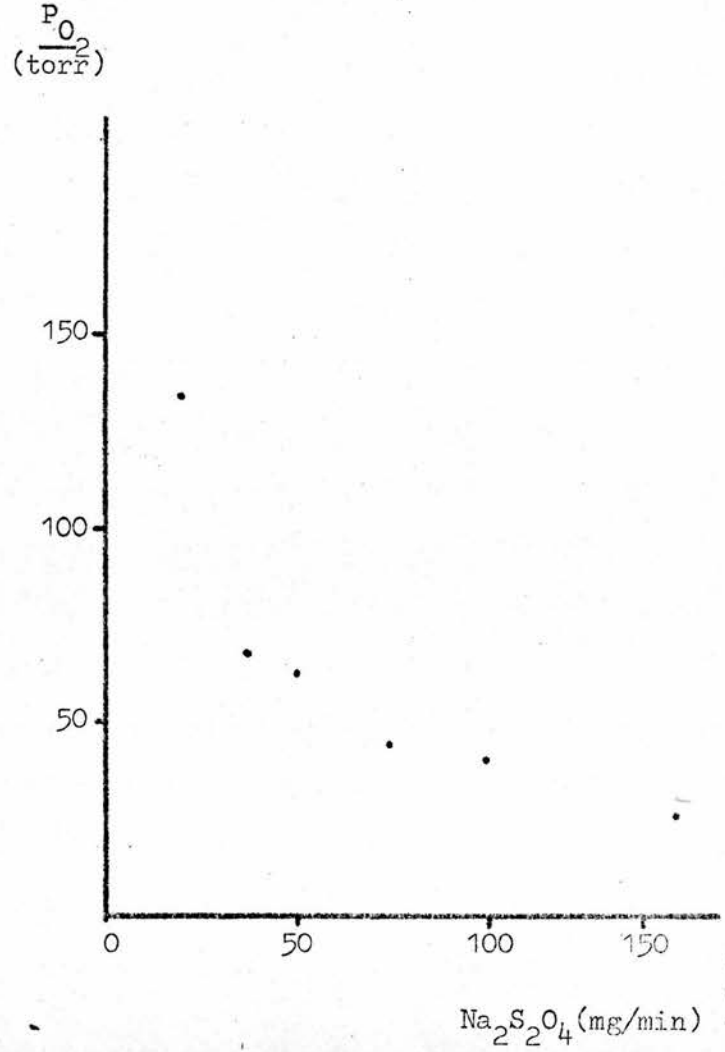
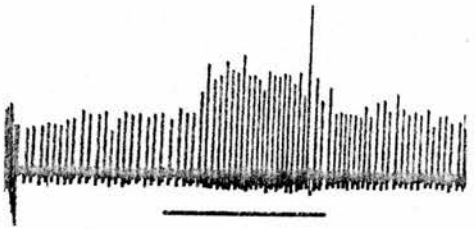


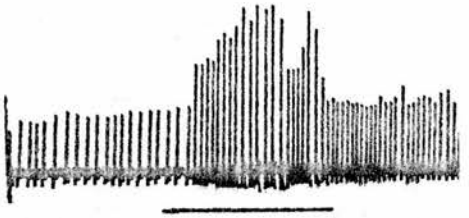
FIGURE 7. THE EFFECT ON PO_2 OF INCREASING RATES OF DITHIONITE INFUSION INTO CAROTID PERFUSATE

INFUSION RATE 20 mg/min PO_2 134 torr

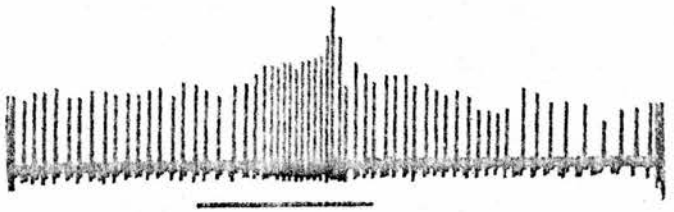


INFUSION RATE 75 mg/min PO_2 44 torr

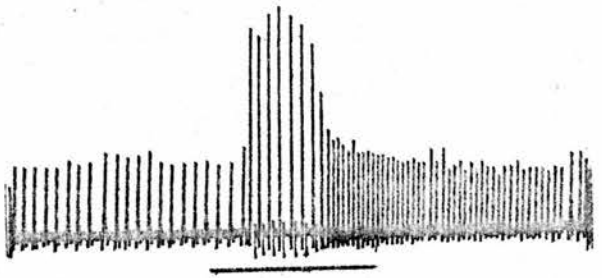
INFUSION RATE 37.5 mg/min PO_2 68 torr



INFUSION RATE 100 mg/min PO_2 40 torr



INFUSION RATE 50 mg/min PO_2 60 torr



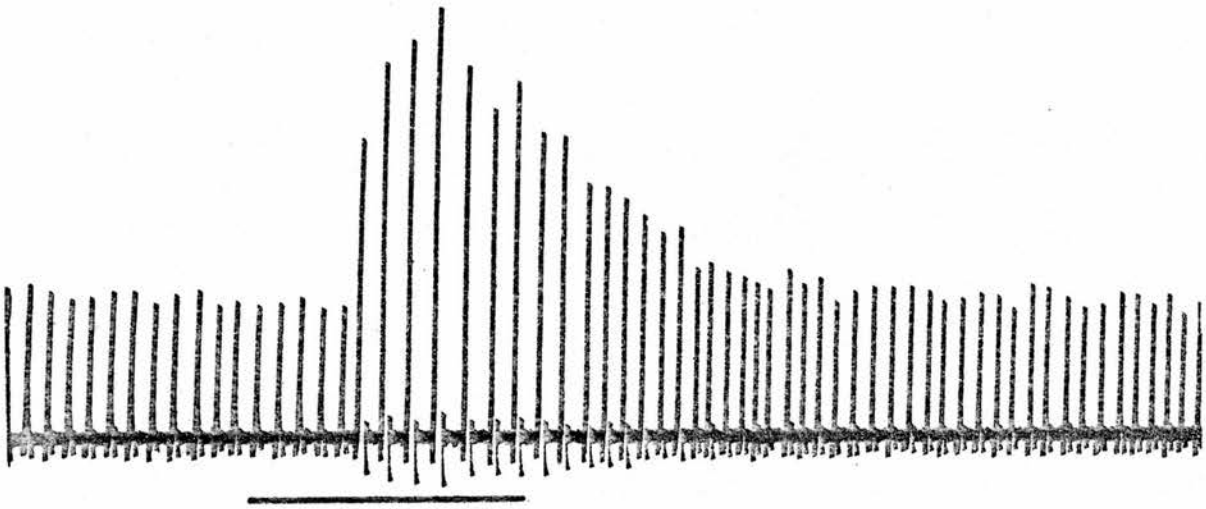
INFUSION RATE 150 mg/min PO_2 25 torr

FIGURE 8.

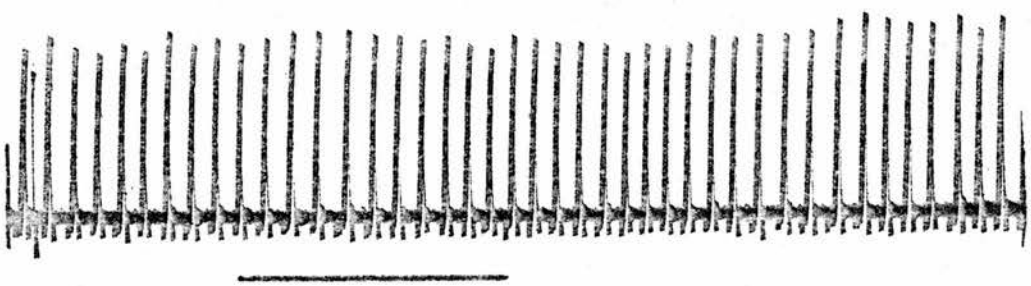
THE EFFECT OF CAROTID BODY DENERVATION
ON THE RESPONSE OF VENTILATION TO DITHIONITE

Infusion rate of dithionite into carotid perfusate = 150 mg/min
P_{O_2} = 6 torr

BEFORE



AFTER



CHEMICAL SPECTROPHOTOFLUORIMETRIC CATECHOLAMINE ASSAY

Objective

Our main objective was to establish a reliable physico-chemical technique in order to assay the noradrenaline and adrenaline of adrenal venous blood. However, we also wanted to extend our method to peripheral plasma and urine samples.

We therefore required an assay which would:-

1. Adequately differentiate between noradrenaline and adrenaline
2. Enable samples to be assayed together in batches of twenty to thirty with consistent recoveries so that we could confidently compare total catecholamine outputs and adrenaline and noradrenaline ratios within each batch. (Assays are bound to vary due to human and technical factors and thus comparisons are more reliable between samples assayed in the same batch).

Each type of sample had its own problems:-

(a) Adrenal Venous Blood

The two catecholamines are present in relatively large amounts with little contamination. Thus only a simple extraction procedure is required and our main concerns were with our ability to differentiate adequately between noradrenaline and adrenaline and with maintaining good consistent recoveries.

(b) Peripheral Blood Plasma

The quantities of catecholamine found in peripheral blood are so small that all the available biochemical techniques are stretched to the limits of their sensitivity.

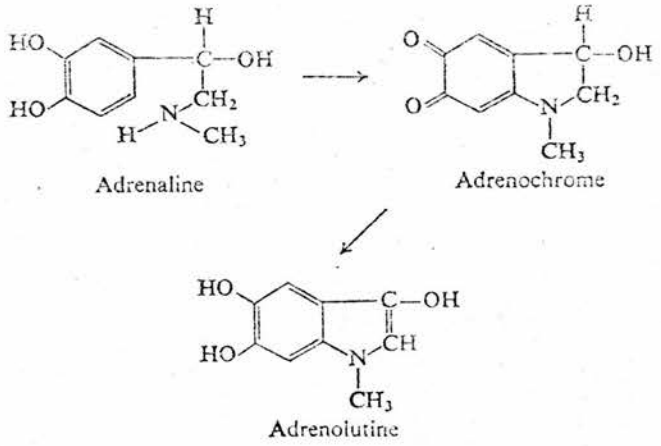
(c) Urine

This is the only situation where closely related compounds to adrenaline, such as their metabolites, are found in quantities significant enough to influence a biochemical assay. Thus purification is very important and required a great deal more attention than it did with the other types of sample.

Introduction

Vulpian initially described the property of the adrenal gland to form lightly coloured compounds and Loew (1918) noted that adrenaline fluoresces in alkaline solutions. Gaddum and Schild (1934) showed that the presence of oxygen was vital for this reaction but their attempts to develop it into an acceptable assay failed due to the transient nature of the fluorescence. A few workers tried to exploit this fleeting fluorescence but success only came when Ehrlen (1948) managed to stabilise the fluorophors with ascorbic acid.

The reaction appears to involve the oxidation of noradrenaline and adrenaline to coloured "chrome" derivatives which are rearranged in alkali to form "lutines". The latter compounds readily oxidise further losing their fluorescence but can be stabilised with an antioxidant such as absorbic acid. This is known as the trihydroxindole (THI) reaction and is so named because of the formation of an indole structure. Most fluorimetric catecholamine assays are based on this sequence and only differ in the choice of oxidising reagent and also the stabilising reagent.



Weil-Malherbe (1952) introduced a similar procedure which involved the condensation of the chrome derivatives with ethylene diamine to form quinoxaline derivatives. When he used the ethylene diamine condensation reaction to assay peripheral plasma he calculated catecholamine levels to be an order higher than those reported using the THI technique. Weil-Malherbe's method was criticised for being less specific than the THI reaction and fell into disrepute (See reviews by Udenfriend and Callingham), although he claimed subsequently to have made improvements so that he obtained results comparable with the more widely used THI method. There is probably little to choose between the two methods for adrenal effluent assay although the condensation reaction introduces another step and thus increases the variability. One advantage of the ethylene diamine condensation method is that the fluorophors are claimed to be very stable which appeals to those workers who intend leaving their samples for long periods before "reading" them in the fluorimeter.

The situation regarding the multitude of fluorescence assays published is well put in Udenfriend's (1965) remark that "there are almost as many variations as there are scientists who use them". This would never have come about if workers had only been interested in assaying

adrenal venous samples and urine. However, the search for a satisfactory peripheral plasma assay has resulted in a plethora of methods which differ only by minor modifications. The catecholamine concentrations found in peripheral plasma are really too low for this technique and it has had to be stretched to the limits of its discrimination.

All the established versions of the trihydroxyindole technique would have been sensitive enough for our adrenal venous sample assay. Our main decision was concerned with the differentiation between adrenaline and noradrenaline and whether the assay was to be based on a one or two reaction tube method.

The former depends on the differences in their fluorescent spectra (as previously described) while the latter involves splitting p145
each sample and exploiting slight differences in the ease of oxidation and stability characteristics of the two catecholamines. Thus it attempts to have fluorescence from only one catecholamine in one of the tubes, and this is usually achieved by oxidising the two samples at different pH's.

Both discussion with other workers in the field and our own experience persuaded us not to use a two tube technique. Although it would theoretically have given a better discrimination, it depends on getting the reaction conditions in each tube "precisely right" and is thus more difficult to use for reliably estimating large batches of samples.

The grounds for choosing our technique were that it had been recommended, it was quite widely used and the various procedures appeared simple and easy to standardise.

The chemical assay of catecholamines has been reviewed in detail by Udenfriend (1958, 1962) and Callingham (1967) and I shall only mention the other THI variants very briefly before discussing our own method.

The following oxidising and stabilising reagents are commonly used in the THI technique:-

Oxidising agents

(a) Manganese dioxide e.g. Lund (1951), Cohen and Goldberg (1957)

(b) Iodine e.g. Crout 1961.

(c) Potassium ferricyanide e.g. Price and Price, (1957) Anton and Sayre (1962), Bertler et al (1958), Vendsalu (1960), Häggendal (1963)

Stabilising reagents

(a) Ascorbic acid (Lund, Anton and Sayre, Price and Price and Crout)

(b) Sulphur containing compounds

1. British anti-Lewisite (BAL)
2. Cysteine Hydrochloride
3. Sodium pyrosulphite.

In addition, in the search for a reliable peripheral plasma assay other factors such as sources of reagents, redistillation, variations in buffer composition etc., have been put forward by various workers for their supposed merits in reducing blank values, or increasing fluorescence or reproducibility.

Nearly all workers emphasise the importance of precise time keeping when adding reagents and reading the fluorescence and also the

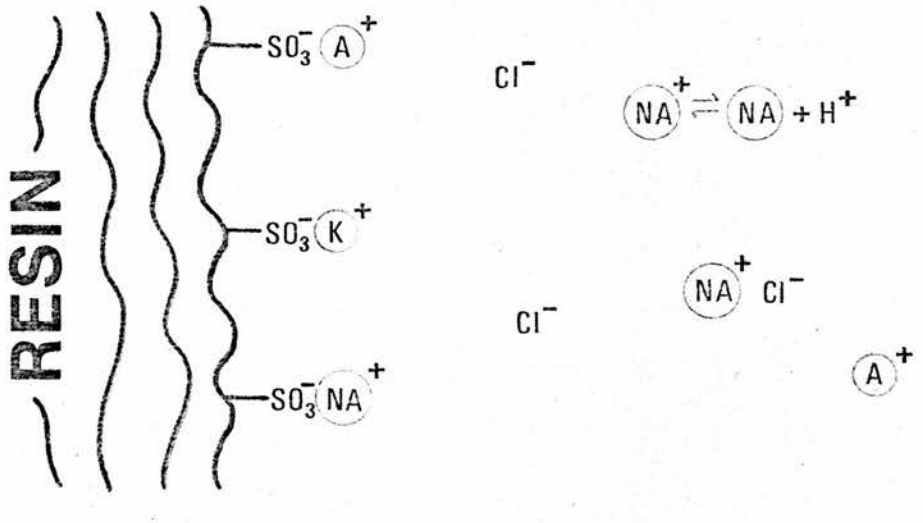
need for adequate washing of glass ware.

We thus used the purest reagents we could afford for the fluorimetry but used Analar grade reagents in the column extraction process (except for final elution of the catecholamines where we used Aristar hydrochloric acid).

OUTLINE OF OUR TECHNIQUE

After separation from the cellular elements of the blood and precipitation of the plasma proteins, the catecholamines were extracted by passage through a cation exchange resin.

The extraction procedure depends on the positively charged nature of catecholamines at around neutral pH which results in them being retained by the negatively charged groups on a sulphonated resin. However, the catecholamines have a similar affinity to any negative ions in the solutions passing through the resin columns.



Thus any anions in solution tend to wash the catecholamines off the columns and it was essential to keep the ionic content of our samples as low and as constant as possible if good and consistent recoveries were to be obtained.

Hydrochloric acid (1N) was used to elute the catecholamines from the columns and the eluate can be stored at this pH or neutralised ready for the fluorimetric estimation.

The fluorimetric reaction used is known as the trihydroxyindole (THI) reaction. Our technique was based on one originally described by Bertler, Carlsson and Rosengren (1958) but slight modifications were made by Vendsalu (1960) and Häggendal (1963). It consists of the following steps. (see diagram on p.122)

1. Oxidation of the catecholamines for three minutes with potassium ferricyanide at pH 6.5.

2. Oxidation stopped and the products stabilised by addition of a sodium sulphite and British anti-lewisite (BAL) mixture.

3. Alkaline rearrangement of the oxidation products for maximum fluorescence with sodium hydroxide (10 N).

4. Samples "read" in the spectrophotofluorimeter by scanning the excitation wavelengths from 300-500 mμ while set at a maximum wave length of 515 mμ.

5. The differentiation between adrenaline and noradrenaline in our technique is based on the different fluorescence spectra of the derivatives of the two compounds. By measuring the unknown's fluorescence intensity at two wave lengths and comparison with standards at these wave lengths, two simultaneous equations are built up and the amounts of the two catecholamines calculated.

Use of native fluorescence

Their phenolic group gives the catecholamines a native fluorescence in the ultraviolet region of the spectrum (285 mμ : 325 mμ) which is maximal at around pH 1. This fluorescence is the same for all catecholamines and is much less intense than that of the derivatives mentioned above. For this reason and the fact that the assays were developed in

the days before instruments could use the "quartz ultraviolet" part of the spectrum, this property has been little exploited. However, we found that it was extremely useful when investigating the column extraction techniques as elution profiles, etc. could be performed by directly reading the fluorescence of the eluates.

Stability of the catecholamines

Catecholamines have been shown to keep for very long periods at pH's between 0 and 4 when deep frozen, (Callingham 1967).

With regard to room temperature, they are stable for days at a pH of 4 or less. Their stability at neutral pH depends on the presence of catalytic ions and oxidising agents, including oxygen. Thus standards and samples waiting to be processed should not be left at room temperature and/or neutral pH. However, our recovery experiments (see p.139 & 267) show that they are stable under the ionic conditions found on the columns. At an alkaline pH, catecholamines oxidise very rapidly, but again this is very dependent on the presence of catalytic ions, oxygen and temperature.

DETAILS OF THE CATECHOLAMINE ASSAY

N.B. The letters underlined in parenthesis refer to the notes which follow this section. P 133

Collection of Samples

Heparinised blood from the left adrenal gland was collected P 94 (as previously described) in a measured volume of 10% sucrose (a) and 0.5% EDTA.

Preparation of the Plasma

Centrifuged immediately at 3,000 r.p.m., 4°C for 10 minutes (b)

The plasma supernatant and packed red cell volumes were recorded and the plasma carefully removed with a syringe and cannula. The plasma was either loaded directly onto the columns (d) or the plasma proteins were precipitated.

Precipitation of the plasma proteins was achieved by thorough mixing with 0.1 volume of 4 N perchloric acid (c). The samples were then frozen (d) and could be stored in this form in the deep freeze.

Neutralisation to pH 4 (e) using 5N Potassium Hydroxide (f). The precipitated proteins had been previously removed by centrifugation at 4°C. After neutralisation the samples were again frozen, thawed and centrifuged at 0°C for 15 minutes.

Extraction and Purification of the Catecholamines

The samples were loaded onto Amberlite CG120 (mesh size 100-200) resin columns (g) of 2 cm length and 0.25 cm. diameter.

The glass columns consisted of a reservoir (20-40 ml capacity) above a narrow bore tube which held the 2 cm column of resin at its lower

end. The resin was converted from the hydrogen to the sodium form (h) prior to loading on of the samples.

Purification (i) was performed by rinsing the columns, after loading on the samples with the following solutions:-

1. H_2O + 0.1% EDTA - 20 ml.
2. Phosphate Buffer (pH 6.5) + 0.1% EDTA - 2.5 ml.
3. H_2O - 5 ml.

Elution (j) was with 4 ml of IN hydrochloric acid (Aristar Grade) into centrifuge tubes (10 ml). The samples were stored in this form (0^oC).

The Spectrophotofluorimetric Assay

Neutralisation of the samples to pH 6.5 (measured on a pH meter) using 5 N potassium carbonate solution.

All the adrenal venous samples were assayed according to the following protocol. Reagents were dispersed manually with Eppendorf pipettes and Repette syringes and mixing was performed mechanically with a "whirleymix".

Assay tubes were prepared as follows:-

1. Samples - Done in duplicate. 0.7 ml aliquots taken from the neutralised samples (l)
2. Standards - Noradrenaline and adrenaline standards (in duplicate) prepared by taking 0.5 ml of standard solution (200 ng/ml in 0.0001 N HCl) and 0.2 ml H_2O.
3. Blanks (s)

   (a) Reagent Blanks - 0.7 ml water

(b) Faded Blanks - 0.7 ml sample to which potassium ferricyanide and sodium hydroxide solutions were added together ten minutes before the BAL/sulphite mixture.

The samples were usually assayed in batches of six (duplicates - twelve tubes) with two pairs of standards and a reagent blank set up in a test tube rack as follows:-

Test No.	
1.	Noradrenaline standard
2.	Adrenaline standard
3.-14.	Samples in duplicate
15.	Noradrenaline standard
16.	Adrenaline standard
17.	Reagent Blank

To each tube the following reagents were added:-

1. 0.1 ml 0.1 M phosphate buffer (pH 6.5)

2. 0.02 ml 0.0025% cupric chloride (p)

Using a stop-clock, the following reagents were added to the tubes according to a strict time schedule. Each reagent was added to the individual tubes at ten second intervals. The size of the batches was thus limited by the number of ten second intervals which could be conveniently fitted into the three minute oxidation reaction time.

1. Potassium ferricyanide (0.25%) - 0.05 ml (n)

2. After three minutes -

   Sodium sulphite (20%) containing 0.5% British Anti-Lewisite (BAL) - 0.125 ml (q)

3. After ten seconds -

   Sodium hydroxide (10 N) - 0.2 ml

In our later experiments i.e. after we had completed our whole animal work:-

4. After four minutes -

Glacial acetic acid - to give final pH 5.0 - 0.22 ml (r)

Fluorescence measurement - After a time delay of at least seven minutes, the contents of each tube in turn were transferred to a clean quartz cuvette and read in the Aminco-Bowman Spectrophotofluorimeter (t) :-

(a) Without acetic acid

Excitation spectrum scanned over range 300-500 mμ

Emission wave length = 515 mμ

N.B. The samples were read as quickly as possible in order to minimise the effects of the gradual fading of the fluorescence.

(b) With acetic acid

Excitation spectrum scanned over range 300-500 mμ

Emission wave length 500 mμ

N.B. The fluorescence was stable for over an hour.

The index of discrimination and the relative fluorescence of adrenaline were both increased when we used an emission wave length of 520 mμ (See p.146 and Figures 11 and 12). p 148 & 149

NOTES ON THE ASSAY TECHNIQUE

The method which I have just described for the quantitative determination of adrenaline and noradrenaline in adrenal venous blood is based on that used by Vendsalu (1960) for the estimation of these amines in samples of human peripheral blood. His technique was based on one worked out by Bertler, Carlsson and Rosengren (1958) for the determination of the two catecholamines in animal tissues. Häggendal (1963) made a number of modifications to Vendsalu's technique and in the following footnotes I shall comment on our method and its deviations from that used by the latter two authors.

Notes on Extraction Procedure

(a) The use of Sucrose rather than 0.9% saline (used by Vendsalu and Häggendal) was found to speed up the centrifugation step (possibly due to erythrocyte cell membrane changes) and did not add to the ionic content of the sample.

(b) Immediate centrifugation followed by refrigeration is essential as loss of catecholamine occurs rapidly in whole blood (11% lost after ten minutes - Mangan and Mason (1958)). However, in plasma, Jones and Blake (1958) found little loss even after twenty four hours at 20-26°C. Iversen (1964) claims that the presence of EDTA also helps to reduce loss.

(c) Omission of the protein precipitation steps was initially tried by Häggendal (1963) who claims that he could run 5-10 ml plasma through the columns without affecting recovery of fluorimetric blank values. However, we found that such volumes of plasma were very slow running on the columns, even after dilution, and the blanks were high and variable. Thus we

were unable to omit the protein precipitation stage when performing human peripheral plasma assays. p 267

However, with the smaller volume of plasma (1-3 ml) and much higher catecholamine content of our adrenal samples, we found that we were able to omit the deproteinisation stage. By washing the sample through with 20 ml H_2O + 0.1% EDTA we achieved a reasonable flow rate and acceptable blank values. In fact the fluorescence spectra in our recovery experiments were indistinguishable whether the catecholamine was washed through the column with water or plasma.

(d) Freezing after the addition of the perchloric acid, appeared to give more complete precipitation of the proteins and it was convenient to store the samples overnight or longer in this form.

(e) Neutralisation to pH 4 at which catecholamines are still very stable. p 128

(f) Potassium hydroxide not only neutralises the acidified samples but also precipitates out most of the perchlorate ions. The samples were refrozen after neutralisation as potassium perchlorate is relatively insoluble at low temperatures.

Freezing was performed rapidly by using an alcohol/solid carbon dioxide mixture and on thawing the samples were rapidly centrifuged at 3,000 r.p.m. for fifteen minutes at $0^{\circ}C$. Removal of the perchlorate ions is essential as the ionic content of the samples must be reduced to a minimum before loading them onto the columns in order to avoid loss of catecholamines from the resin*. The samples were frozen twice because omission of the first freeze (i.e. that after protein precipitation) resulted in a faint opalescence which was not removed by the second freeze

*see p.126

(at neutral pH). This was not inconvenient as the samples were usually stored in the freezer after the addition of the perchloric acid.

I found that neutralisation at this stage with potassium carbonate (as used by Vendsalu and Häggendal) usually lead to bubbling on the resin column which stopped the flow. Furthermore, the gradual loss of carbon dioxide while the samples were in the reservoirs above the columns resulted in a slow rise in pH, which would increase the susceptibility of the catecholamines to oxidation. However, both these problems were avoided by neutralising the samples with a carefully measured volume of potassium hydroxide (5N) and the final end point was achieved by dropwise addition of 0.5N potassium bicarbonate which has a near-neutral pH. A tiny magnetic stirrer was used to prevent the formation of regions of local alkalinity during the neutralisations.

(g) Choice of extraction technique. We employed a strong cation exchange resin of the sulphonic acid type. The other widely used extraction technique involves the absorption of the catecholamines onto alumina by either a batch or a column procedure and appeared to have a number of disadvantages. Firstly, most workers recommended a tedious preparation process for the alumina and each batch has to be tested for its recovery properties. Secondly, the absorption stage requires an alkaline pH and although performed in the presence of the antioxidants, this is very likely to increase the variability of the recoveries. Finally, most authorities considered that when using alumina, better results are obtained with the batch rather than the column approach, although the former is inherently more tedious and exacting. Thus, as there appeared to be little to choose between the alumina and ion exchange techniques regarding published recovery figures and as the reliability of any

procedure must be in part inversely dependent on the number of exacting steps, we decided upon the latter technique.

The resin chosen was Amberlite CG 120 (- recommended by Häggendal) and is equivalent to the Dowex 50 which was employed by Bertler et al and Vendsalu. The resins can be used in either the hydrogen or the sodium form but Häggendal claims that the latter gives better recoveries. This is possibly because the change in resin size during elution helps to "dislodge" the catecholamines.

We used a more coarse form of resin (i.e. 100 - 200 mesh) rather than the 200 - 400 mesh used by Vendsalu and Häggendal. Our mesh size gave a very satisfactory flow rate by gravity alone and thus did not require pumping devices to aid the passage of solutions through the columns (e.g. Bertler et al). Such devices require constant supervision to prevent air being forced into the columns when all the solution has run through. However, with our technique, surface tension stopped the flow before the columns ran dry.

Two problems which Häggendal discussed are the presence of fluorescent contaminants in the resin and loss of catecholamine during passage through the columns. The fluorescent contaminants can be eliminated by careful preparation of the resin. The fines are removed by repeated washes with water. This is followed by recycling the resin several times between the sodium and hydrogen forms so that alternate expansion and contraction of the resin helps to expel impurities.

Häggendal found that the loss of catecholamines on the columns is a function of their length. Shorter columns are also faster flowing

and require a smaller elution volume. We found that the recovery of standards from our short (2 cm) columns was over 90%. (see fig 9) Further shortening of the columns would have decreased the required elution volume but greatly increased the chance of catecholamine loss if the salt content of the sample was high (see p.126, 139 & 267).

(h) Preparation of the columns The resin was stored in the hydrogen form. The columns consisted of 2 cm of resin retained by quartz wool and were prepared by running through the following solutions:-

(i) 10 ml H_2O - to wash off any excess acid

(ii) 10 ml Phosphate Buffer (pH 6.5) - converted resin to the sodium form (N.B. final effluent pH = 6.5 - checked with Litmus paper).

(iii) 10 ml H_2O + 0.1% EDTA to remove divalent metal ions

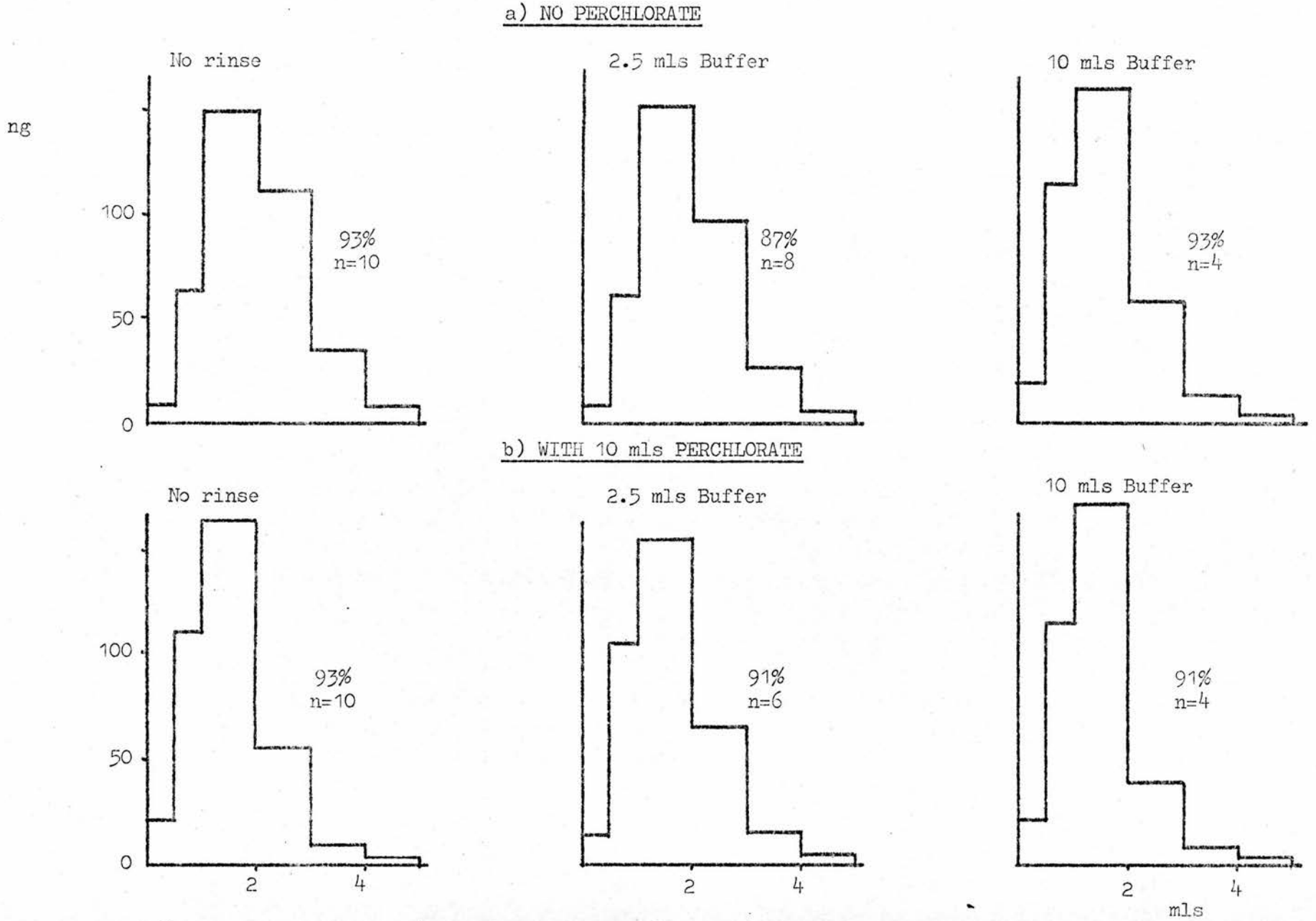
(i) Rinsing to remove contaminants No more than 5 ml of Phosphate Buffer was used at this stage although recovery experiments showed that up to 10 ml buffer could safely be used without resulting in a detectable amount of catecholamine being washed off the columns. (See Figure 9). p13 The final rinse with water did not contain EDTA in case its presence affected the fluorimetry. (However, when fluorimetry was performed on standards using phosphate buffer with and without 0.1% EDTA no effect could be detected).

(j) Elution The volume of 1N hydrochloric acid chosen (4 mls) had been shown by elution profiles and recovery experiments (see Figure 9 and p. 267) to be adequate for virtually complete elution. More concen trated acid can be used to elute into a correspondingly smaller volume but we found this to disproportionately increase our blank values - (presumably due to contaminants washed off the columns).

Häggendal recommended discarding the first couple of drops of eluate claiming that this contained a high proportion of the contaminants and no catecholamine. However, we did not find that this reduced our blank value and it was very risky as the catecholamines appeared at a variable point in the elution profile which depended on the salt content of the sample. Also, it was easy to forget to start collecting!

There is a difference of opinion on the subject of whether it is essential to elute without interruption or whether it is better to elute in stages and thus give time for the catecholamine to diffuse out of the resin. We found no difference between these two methods.

FIGURE 9. EFFECT OF PHOSPHATE BUFFER RINSE ON ELUTION PROFILE OF ADRENALINE AND NORADRENALINE STANDARDS
(Fractional elution from 2cm Amberlite CG-120 with 1N HCl)



% Recovery shown beside each elution profile

Notes on the Fluorimetric Technique

(k) Oxidation pH A pH of 6.5 was chosen as, at this pH, the derivatives of the two catecholamines have about equal fluorescence and fluctuations of about 0.5 pH units make negligible difference to the final fluorescence.

Prior to oxidation the acid eluates are carefully neutralised with 5N potassium carbonate using a pH meter. With practice, the calculated neutralising volume can be pipetted out with safety but the pH should not be allowed to exceed about 7.5. Potassium bicarbonate would have been much easier to use but it is far less soluble and thus its use would have involved a much greater dilution factor.

(l) Use of duplicates Samples and standards were always in duplicate in each batch in case a tube was dropped and also because it was encouraging to watch the two traces being drawn on top of (or very near to) each other! This is one of the ways to keep up interest in a tedious assay and thus maintain good results. Such considerations are vital for success in an assay technique that depends on an important human contribution. Therefore, I have tried to avoid tricky manipulations, etc. and make each step as simple and "fool proof" as possible.

(m) Timing The importance of very accurate timing during all stages of the fluorometric procedure has been emphasised. Before the introduction of the automated technique described later, we achieved this by using "Repette" syringes to rapidly deliver consistent volumes of each reagent and timed ourselves with a stop-clock. The technique was very reliable but required the presence of an assistant, unless small

batches of tubes (less than twelve) were used. With an assistant, the maximum number of tubes per batch (not including blanks) was eighteen. Reagents were added to each tube in turn every ten (two operators) or fifteen (single operator) seconds and thorough mixing was performed after the addition of each reagent by the use of a "Whirlymix".

(n) Potassium ferricyanide Most workers preferred this oxidising agent, as iodine, the only other widely used agent, takes much longer to oxidise the catecholamines and the reaction appears to be greatly influenced by the laboratory lighting conditions, (Callingham 1968).

(ρ) Use of Copper Ions Throughout our work cupric ions were added prior to oxidation. Bertler et al used zinc ions as a catalyst but Vendsalu observed that this resulted in the final solution becoming cloudy (probably due to the formation of zinc hydroxide), and thus necessitating centrifugation before reading. Later workers (Sandhu and Freed) also encountered this problem with zinc.

Häggendal used cupric ions claiming that they accelerated the oxidation process and increased the stability and intensity of the fluorescence. He also comments that a weakening of fluorescence occurs in alkaline solution due to the formation of complexes between the cupric ions and the lutines but this is eliminated when BAL/sulphite is used as an antioxident. (He presents little evidence to support these remarks). Valori et al confirmed that cupric ions had a catalytic effect but mainly in relation to the oxidation of adrenaline.

We investigated the effect of cupric ions by running standards with and without their presence and could show no significant difference. However, they definitely do have a catalytic effect under some circum-

circumstances because they were essential for the oxidation of adrenaline at a low pH (around 3). Furthermore, we found that they made a great difference to the amount of fluorescence obtained when the THI. reaction was employed with the metanephrines. (See p.281)

Thus as we had shown that they did no harm and knowing that metal ions play some part in the oxidation of catecholamines and their derivatives, we always added a small volume of cupric solution prior to the oxidation.

(q) Stabilisation of the Lutines Much previous work has been done on this aspect of the reaction. Ascorbic acid was widely used by many workers including Vendsalu but he noted that it contributed considerably to the blank value in a time dependent manner. (Dr. T.B.B. Crawford says that this is due to impurities in the ascorbic acid and thus he keeps a special supply).

Häggendal investigated the use of many other agents and found that the best results were obtained with sulphur containing compounds. A solution of dimercaptopropanol (BAL) in sodium sulphite gave the most reproducible results with lower and more stable blank volumes without reducing the fluorescence of the lutines. In the presence of BAL the shape of the fluorescence spectra was altered to give two peaks instead of the single peak obtained with ascorbic acid. Häggendal considered that ascorbic acid obscured the peak at the lower wave length. These two peaks were very helpful for identifying the catecholamines and served as what can best be described as a "quality control" for the fluorimetric reaction. However, the best wave lengths for the differential equations were on either side of the second (original) peak.

It is important to avoid contact between solutions containing BAL and other reagents involved in the T H I reaction in order to prevent premature interference with the oxidation. Furthermore, BAL is somewhat unpleasant to handle and should be kept in a fume cupboard.

Valori et al (1970) found that solutions of BAL in sodium sulphite were often cloudy which lead to what they described as "quenching" (a term loosely used by many writers) and also variability in the blanks. We also observed this cloudyness but found that it could be minimised by vigorous mixing of the two reagents (BAL is not very soluble) and by using fresh solutions which were not more than two hours old. Reducing the concentration of BAL also prevented this effect but as a consequence, the fluorophors were less stable and the standards showed more variability. Valori and his colleagues investigated various reagents which would increase the solubility of BAL in water in order to use a more concentrated solution and so dispense with other antioxidants. They describe a technique in which a BAL-formaldehyde solution is used followed by glacial acetic acid to bring the final pH of the mixture to 5.3. We found that their stabilising mixture rapidly became very dirty, produced poor fluorescence and used up large quantities of BAL. However, it gave us the incentive to investigate the effect on the final fluorescence of adding glacial acetic acid - the use of which had been previously suggested by Dr. T.B.B. Crawford.

(r) Use of Glacial Acetic Acid Using our standard fluorimetric reaction we added glacial acetic acid in 0.05 ml aliquots to the final mixture and observed a shift in the fluorescence spectra. The first peak was greatly diminished for both catecholamines. However, the second peak for noradrenaline was reduced by about 20% without affecting

the adrenaline peak. The alteration in the relative sizes of the second peaks (previously NA had been higher) greatly increased the discrimination of our assay. (Compare the diagrams of the fluorescence scans, Figures 10-12). The shift was complete at pH 5 and at this pH a change of 0.5 pH units either way had negligible effect. Furthermore, unlike our previous alkaline fluorescence mixtures which faded with time (this was very variable but was by about 20-30% over 45 minutes) the acidified samples could be read repeatedly over half an hour and showed little tendency to fade.

(s) Blanks Background fluorescence from the reagents and from any contaminants which were not removed by our purification procedure, contributed to the measured fluorescence of our samples. Thus a "blank" value was required in order to determine the fluorescence due solely to the catecholamines.

A reagent blank is prepared for calc ulations with the standards. For the sample blank, the fluorescence of the catecholamines must be prevented without omitting any of the reagents. There are two possible approaches:-

1. Non-oxidised blanks - The antioxidant/stabilising mixture (BAL) sulphite is added before the oxidising solution (ferricyanide).

2. Faded blanks - The lutines are allowed to undergo further oxidation to non-fluorescent derivatives. Oxidant and alkali are added and the sample left for at least 10 minutes. Thus the catecholamine fluorescence has "faded" by the time the antioxidant/stabilisation mixture is added.

We found that the non-oxidised blanks showed catecholamine fluorescence peaks presumably due to spontaneous oxidation during the neutralisation of the column eluates. Häggendal suggested removing an aliquot from the eluate prior to neutralisation in order to obtain satisfactory non-oxidised blanks. However, it was simpler to use faded blanks and we used these throughout our assays.

(t) Fluorescence reading and calculation

The intensity of the fluorescence emitted by the sample was displayed on the linear scale of the Aminco-Bowman Photomultiplier Microphotometer. The scale is from 0 to 100 and is multiplied by the setting on the Meter Multiplier switch which was calibrated in steps from 0.001 (--0.003, 0.01,--) to 1.0. The latter switch was altered for each individual sample's fluorescence so as to give the maximum possible reading on the 0-100 scale. This minimised the error in reading the meter for if the scale can be read to, say, one division, then the error in the reading at 100 is 1% while at 10 it is 10%.

In our set-up, the meter was linked to a Servoscribe pen recorder which followed the deflection of the meter (The paper chart was conveniently graduated from 0 to 100). The chart drive was synchronised to the scanning motor switch on the Aminco-Bowman so that the paper only moved when the scan was in progress. The scans were commenced at precisely 300 mμ and the pen was always lined up with one of the heavy (1 cm) lines on the Servoscribe paper. As the speed of the chart drive and wave length scanning motors were never altered, we could determine the wave length of each position on a fluorescence trace by its distance from the start of the scan. (see tracings of scans-Figures 10-12).

We always superimposed the noradrenaline and adrenaline standard traces on top of one another as this enables us to easily assess the degree of differentiation. When choosing the two positions to read the fluorescence intensity for the differential calculation, I tried to keep a balance between obtaining the greatest possible ratio between the two catecholamines and:-

1. Having a reasonably high intensity of fluorescence above the blank value.
2. Avoiding interference from the scatter peak which was around the emission wave length and became relatively larger as the catecholamine content of the samples decreased.

The wave lengths chosen for each set of fluorimetric conditions are shown in their respective fluorescent scans (Figures 10-12).

In my discussion I have used the concept of "Index of Discrimination" by replacing "activity" with fluorescence intensity (see Gaddum, 1959) to compare our variant of the fluorimetric assay with the techniques on which it is based.
(i.e. Fluorescence intensity of A ÷ NA at the higher wave length ÷ the ratio at the lower wave length).

For the three sets of fluorimetric conditions which we used, the Indices of Discrimination were:-

(a) No Acetic Acid: I. of D. = 4

(b) Acetic Acid with emission wave length 500 mμ: I. of D. = 8

(c) Acetic Acid with emission wave length 520 mμ: I. of D. = 13

FIGURE 10.

FLUORESCENCE SPECTRA OBTAINED WITH 100 ng STANDARDS OF A AND NA AS USED IN DIFFERENTIAL ASSAY OF ADRENAL VENOUS BLOOD SAMPLES.
Excitation scan (300 - 500 mμ) of lutines at pH > 10 and emission wavelength 515 mμ.

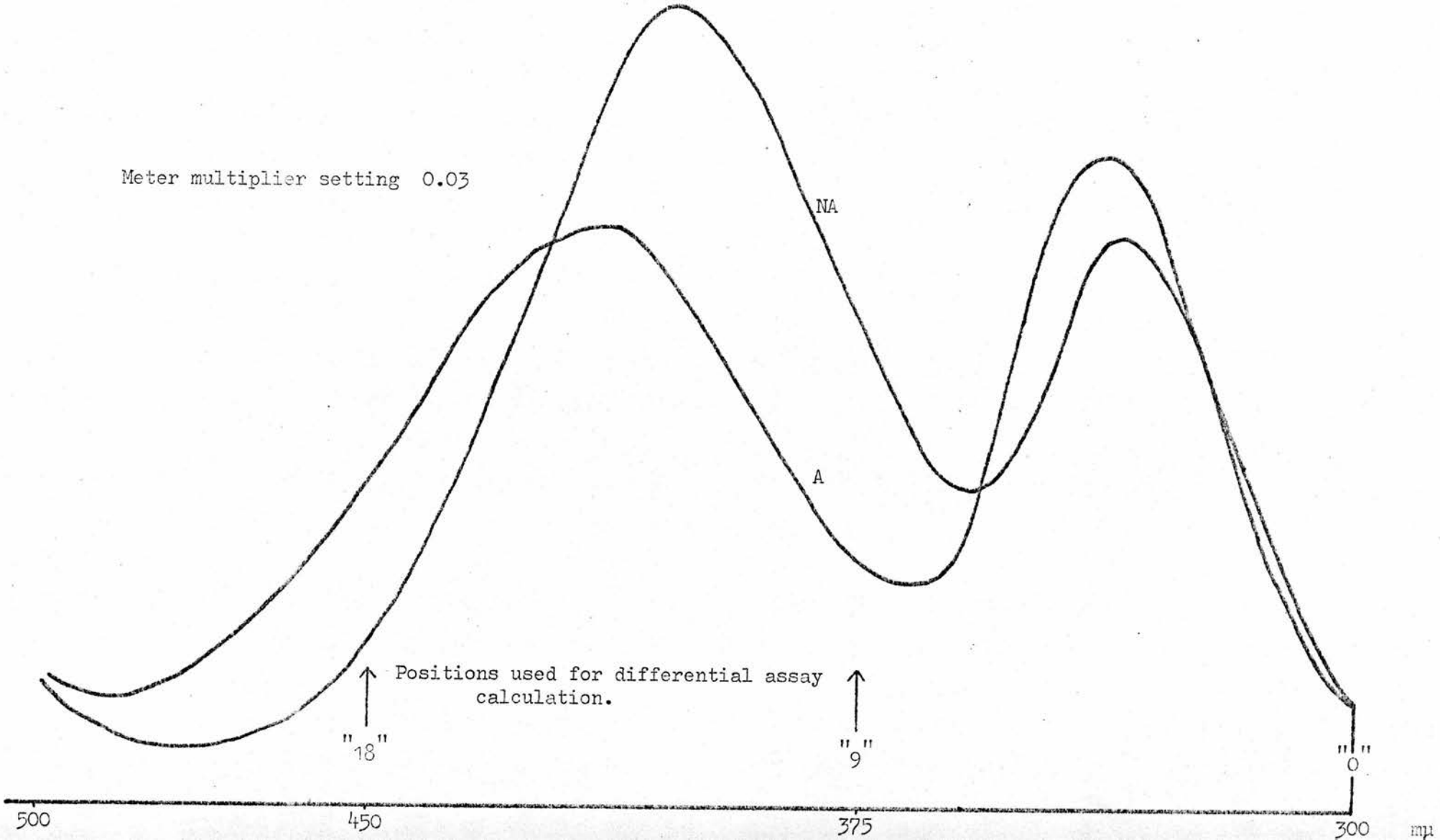


FIGURE 11.

TRACING OF FLUORESCENCE SPECTRA ALSO USED IN AUTOMATED ASSAY, WHICH GAVE I of D = 12.

Excitation scan 300 - 500 mμ
Emission wavelength 520 mμ
pH 5.0 (after addition of glacial Acetic acid)

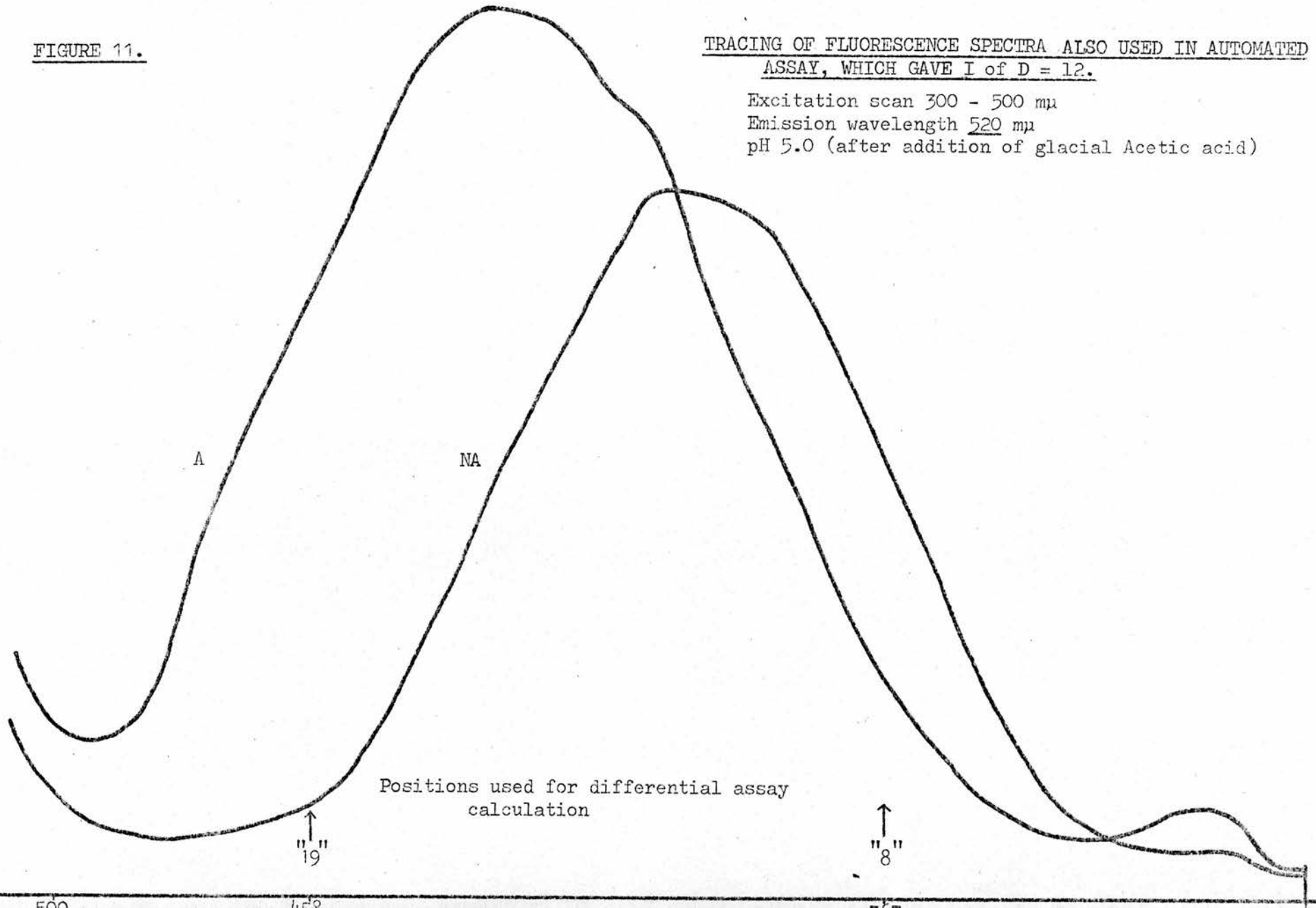
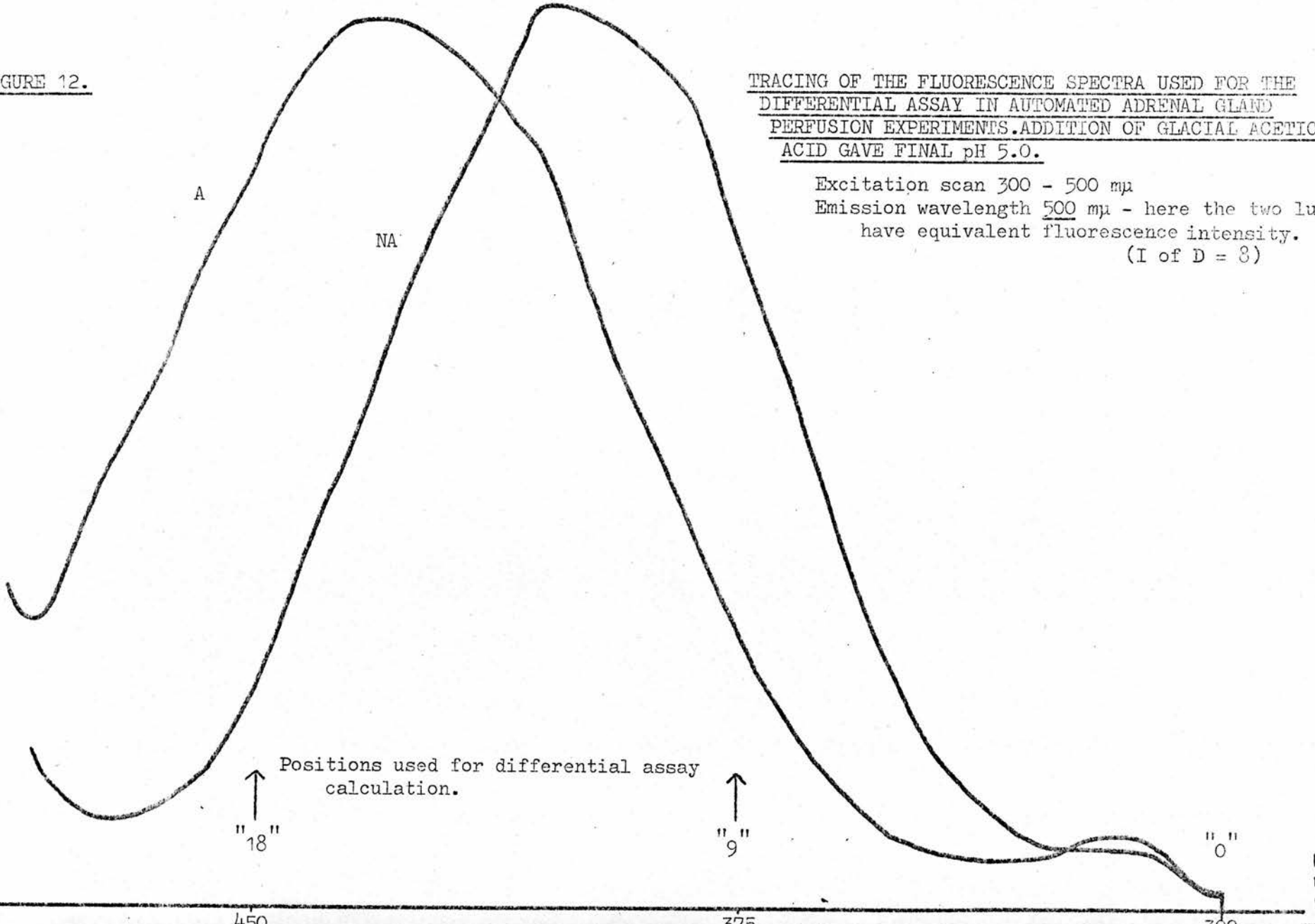


FIGURE 12.

TRACING OF THE FLUORESCENCE SPECTRA USED FOR THE DIFFERENTIAL ASSAY IN AUTOMATED ADRENAL GLAND PERFUSION EXPERIMENTS. ADDITION OF GLACIAL ACETIC ACID GAVE FINAL pH 5.0.

Excitation scan 300 - 500 mμ
Emission wavelength 500 mμ - here the two lutines have equivalent fluorescence intensity.
(I of D = 8)



Calculation

To calculate the NA and A contents of the mixture, two assumptions are made:-

(a) The relationship between intensity of fluorescence and concentration is linear.

(b) The fluorescence of the two catecholamines is additive.

N.B. The previous workers in the field and our own standard curves show that these assumptions are justified for the range of concentrations with which we were concerned.

For a catecholamine mixture a pair of simultaneous equations can be built up using the standards and from these equations the amount of NA and A present is calculated. (See Bertler et al 1958).

We wrote and used an Olivetti programme to solve these equations when analysing our samples.

Discrimination of our assay

An appreciation of the ability of our assay to discriminate between noradrenaline and adrenaline can be obtained by considering:-

1. The assay of known mixtures of standards.

2. The comparison of our technique with those from which it was derived.

1. Mixtures of standards

Two examples are given in which we used the technique without Acetic Acid and thus the I.O.D. was four.

	MIXTURE		CALCULATED	
n	A	NA	A	NA
4	90	10	91.1 ± 0.5	7.3 ± 1.5
4	70	30	71.3 ± 1.5	27.0 ± 1.8
4	50	50	52.8 ± 1.1	49.8 ± 0.9
4	30	70	27.2 ± 2.4	67.8 ± 2.8
4	10	90	11.5 ± 2.0	88.4 ± 2.3
10	90	10	88.5 ± 1.4	9.2 ± 1.0
8	80	20	78.1 ± 2.3	19.6 ± 1.1
11	70	30	68.2 ± 1.8	27.9 ± 1.1

(Mean ± S.D.)

2. Comparison with other techniques

All the three publications on which we based our assay give examples of their standards and blanks and from these I have calculated an I.O.D. for each case. p146

(a) Bertler et al

Wavelengths = 410/540 and 455/540 I.O.D. = 5

(b) Vendsalu

Wavelengths = 410/540 and 455/540 I.O.D. = 4

(c) Häggendal

Wavelengths = 400/515 and 450/515 I.O.D. = 7

Häggendal quotes uncorrected instrument values for his wave lengths. From a comparison of his fluorescent ratios with our curves it appears that his spectrophotofluorometer was calibrated slightly differently to our machine and he was achieving a higher I. of D. by taking his second reading at a longer wavelength. However, as a consequence, the fluorescence was reduced and closer to the blank value.

ISOLATED ADRENAL GLAND PERFUSION EXPERIMENTS

Introduction

We decided to investigate whether our whole animal studies were supported by the results obtained using isolated perfused adrenal glands.

Firstly, there appeared to be a species difference between the dog and the cat with respect to the distribution of muscarinic receptors in the adrenal medulla in parallel with the physiological difference we had already investigated with regard to the baroreceptor and chemoreceptor reflexes. Thus we compared the responses of dog and cat adrenal glands to specific muscarinic and nicotinic stimulant drugs. We chose acetyl β methyl-choline as our muscarinic drug and meta-hydroxy phenylpropyl trimethylammonium (hpp TMA) which Dr. R.B. Barlow recommended as being a very specific nicotinic agonist. p 63

Secondly, if we could show that corticosteroids, in the concentrations expected within the adrenal medulla during stress, released catecholamines, this would be a crucial piece of evidence in favour of a humoral mechanism participating, via the anterior pituitary - adrenocortical axis, in the control of the secretion of adrenal catecholamines.

Source of Glands

The animals were heparinised prior to removal of the glands which were cannulated in a retrograde fashion via the adrenolumbar vein. It was acceptable to use a very small cannula for cannulating the adrenolumbar veins in the cats because the high resistance was now sited upstream of the gland. p16

The glands were either used that evening or stored overnight in refrigerated ($4^{o}C$) Locke's solution. In the latter case, they responded better to drugs if they had been perfused for 10-20 minutes prior to storage.

Perfusion

The glands were perfused with phosphate buffered Locke's p 165 solution at a constant flow of 1 or 2 mls/minute delivered by a Watson-Marlow Pump. The Locke's solution was oxygenated and maintained at $37^{o}C$ by passage through a heating coil connected to a water bath. A pump circulated water through the coil and a jacket which surrounded the gland. (The jacket and coil were taken from a Langendorff isolated heart perfusion apparatus). The gland was suspended in a small glass funnel (surrounded by the heating jacket) from which the effluent dripped.

In the early experiments, the effluent was collected by hand for one minute intervals and the gland perfused at 1 ml/minute.

The main series of experiments used the on-line automated assay system which we had developed. The gland was perfused at a flow of 2 mls/minute and samples were collected for 30 second periods by using the moving conveyor belt of the Unicam AC60 Chemical Processing Unit.

Drug Infusions

The gland was initially allowed to equilibrate with the perfusate over a period of $1\frac{1}{2}$ to 2 hours. The drug tests were then commenced with infusion times lasting between 1 and 10 minutes. In each case sequential samples were collected before, during and after the drug infusion at either thirty second intervals or continuously depending on the duration of the drug infusion.

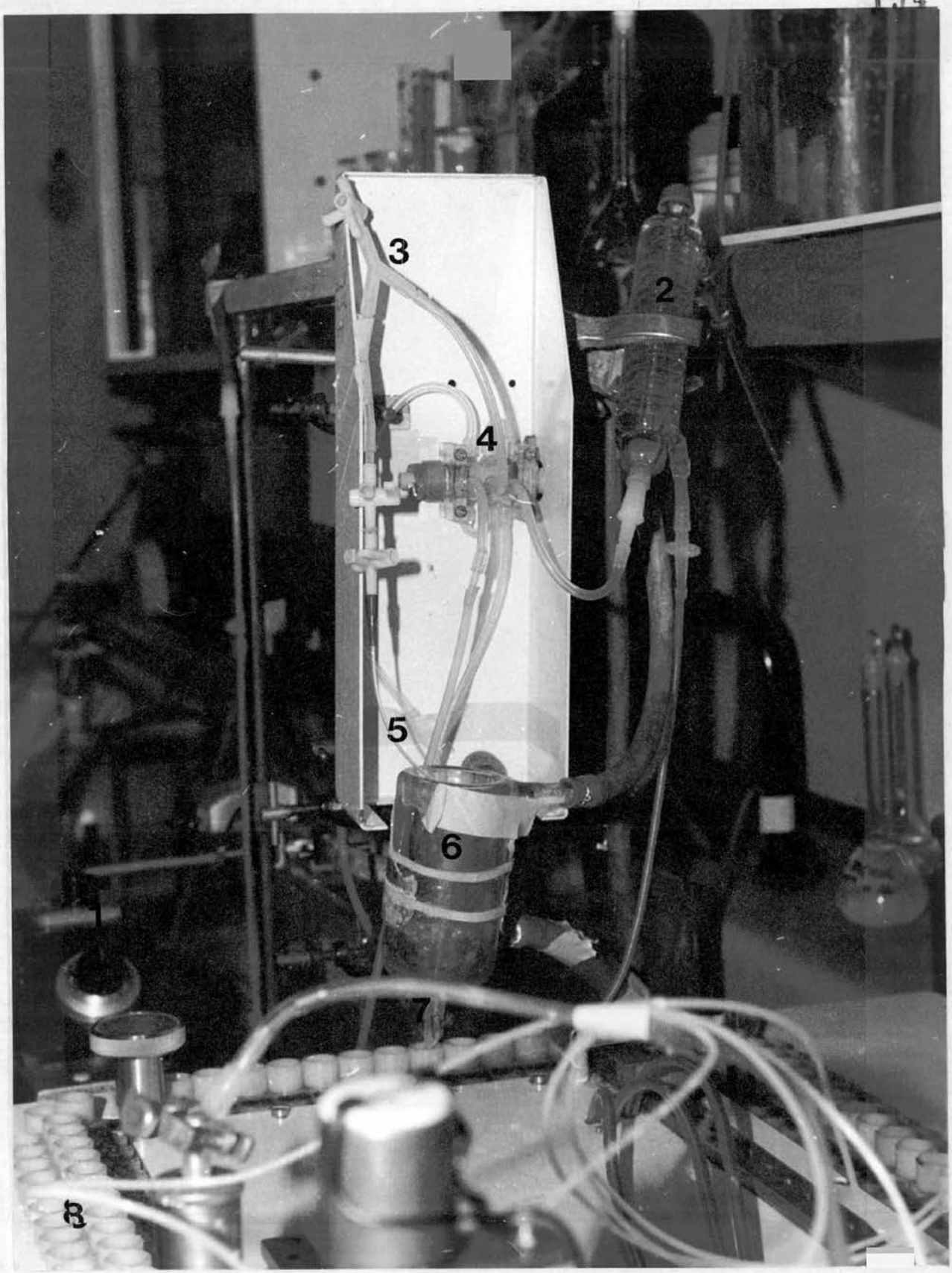


FIGURE 13.

ADRENAL GLAND PERFUSION SYSTEM

1 Watson Marlow pump
2 Heating coil
3 Bubble trap
4 Multitap - for perfusate change over
5 Cannula to gland (tied into adreno-lumbar vein)
6 Heating jacket
7 Funnel with portex tubing end piece
8 Track of AC 60 - holds sample collection tubes

NB This is the earlier arrangement when the Watson Marlow pump was sited upstream of the multitap.

The drugs were made up in Locke's solution, oxygenated and heated to 37°C in the water bath prior to and during the infusion. The change-over from perfusion with Locke's solution alone to that containing a drug was achieved rapidly and smoothly by means of a single lever action gang of three-way taps interconnected by a system of short tubes. (This tap arrangement was taken from a semi-automatic reagent delivery device - see photograph, Figure 13). The mechanism ensured that there was no interruption in the flow through the gland when the perfusate was changed. Periodically we performed control changeover tests using Locke's solution that did not contain drugs in order to confirm that the actual change-over itself did not provoke a release of catecholamines from the gland.

At least 15 minutes was allowed to elapse between individual drug infusions so that the gland had returned to a steady resting output. After repeated drug infusions a gradual fall off in the responsiveness of the glands was observed. This varied from gland to gland but was also reflected in a progressive reduction in the resting output.

Drug Infusion Protocol

(a) Studies on Selective Release

The following solutions were infused into both the dog and the cat glands.

1. Acetyl β Methyl Choline (Methacholine)
   Concentrations:-
   Dog: 10^{-8} - 10^{-6} Molar
   Cat: 10^{-6} - 10^{-4} Molar

2. M-hydroxyphenylpropyl trimethyl ammonium iodide (hpp TMA)
   Concentrations:-
   Dog: 10^{-8} - 10^{-5} Molar
   Cat: 10^{-7} - 10^{-5} Molar

3. Carbachol

Concentrations:-

5×10^{-6} - 10^{-4} Molar

N.B. only used with cat glands.

All three drugs were infused for three minutes and the 30 second sample collections taken before, during and after the drug infusions.

Collection Schedule

(Time (mins)	Drug Infusion	Collection
0		Control
$\frac{1}{2}$		-
1		Control
$1\frac{1}{2}$		-
2		Control
$2\frac{1}{2}$		-
3		Control
$3\frac{1}{2}$ ⟶ 4	START	- TEST
$4\frac{1}{2}$		TEST
5		TEST
$5\frac{1}{2}$ ⟶ 6	STOP	TEST Recovery
$6\frac{1}{2}$		Recovery
7		-
$7\frac{1}{2}$		Recovery
8		

Blocking Drugs

In the work on cat glands either Atropine (3×10^{-5} Molar) or Hexamethonium (3×10^{-4} Molar) was added to the perfusion fluid in the second part of the experiment and, after allowing at least 30 minutes for equilibration, a series of drug tests was performed as above. The agonist drugs were also made up in the Locke's solution which contained the blocking drug.

(b) Studies on the humoral mechanism

These studies were performed only on dog glands. All collections were made for 30 seconds with 30 second intervals between collections. The following drugs were infused:-

1. Hydrocortisone sodium succinate (Efcortelan - Glaxo)

   Concentration:-

   25 ug/ml - 200 ug/ml
   Infusion time - 8 mins.
   Collections - 4 controls
   8 tests
   3 recovery

2. Aldosterone (Aldocorten - Ciba)

   Concentration:-

   5 ug/ml - 20 ug/ml
   Infusion time - 5 to 10 mins.
   Collections - 4 controls
   5 - 10 tests
   3 recovery

3. Deoxycorticosterone

   Concentration:-

   40 ug/ml - 100 ug/ml
   Infusion time - 8 minutes
   Collections - as for Hydrocortisone

AUTOMATED GLAND ASSAY

Our initial work with isolated perfused adrenal glands was done entirely by hand. Both the sample collection and fluorimetry was done with "one eye on the stop-clock" while the drug - perfusate change-overs involved turning a number of plastic three-way taps simultaneously (we had not introduced the single action change-over device). The technique was tedious and unless there were two or three participants, each drug test took over an hour or else the samples had to be stored in the freezer for assay at the end of the experiment.

Apart from the obvious frustrations of this approach, the long delay between drug test and result meant that the drug infusions had to be done blind. Both, matching doses of different drugs so as to give releases of equal magnitude and producing dose-response curves, were very difficult.

A few automated techniques had already been published (e.g. Fiorica (1965), McCulloch (1968)) but these were all based on continuous flow systems such as that used in the Technicion "Auto-Analyser". In these, the samples are separated by bubbles as they move along a pipe into which reagents are fed at intervals and then pass through a flow-through cuvette. It is easy to use this type of system when the reading of fluorescence is made only at the one wave length (i.e. to determine the total catecholamine content of each sample). However, a continuous flow system is unsuitable in a technique in which a scan has to be performed or two wave lengths read as was necessary for our differential assay. Thus for estimating the two catecholamines individually, automated methods usually involve splitting the sample and make use of

two different oxidation pH's or stabilising reagents to prevent fluorescence of one or other catecholamine (e.g. Merrils 1963). I have already commented on the disadvantages of the latter types of technique.

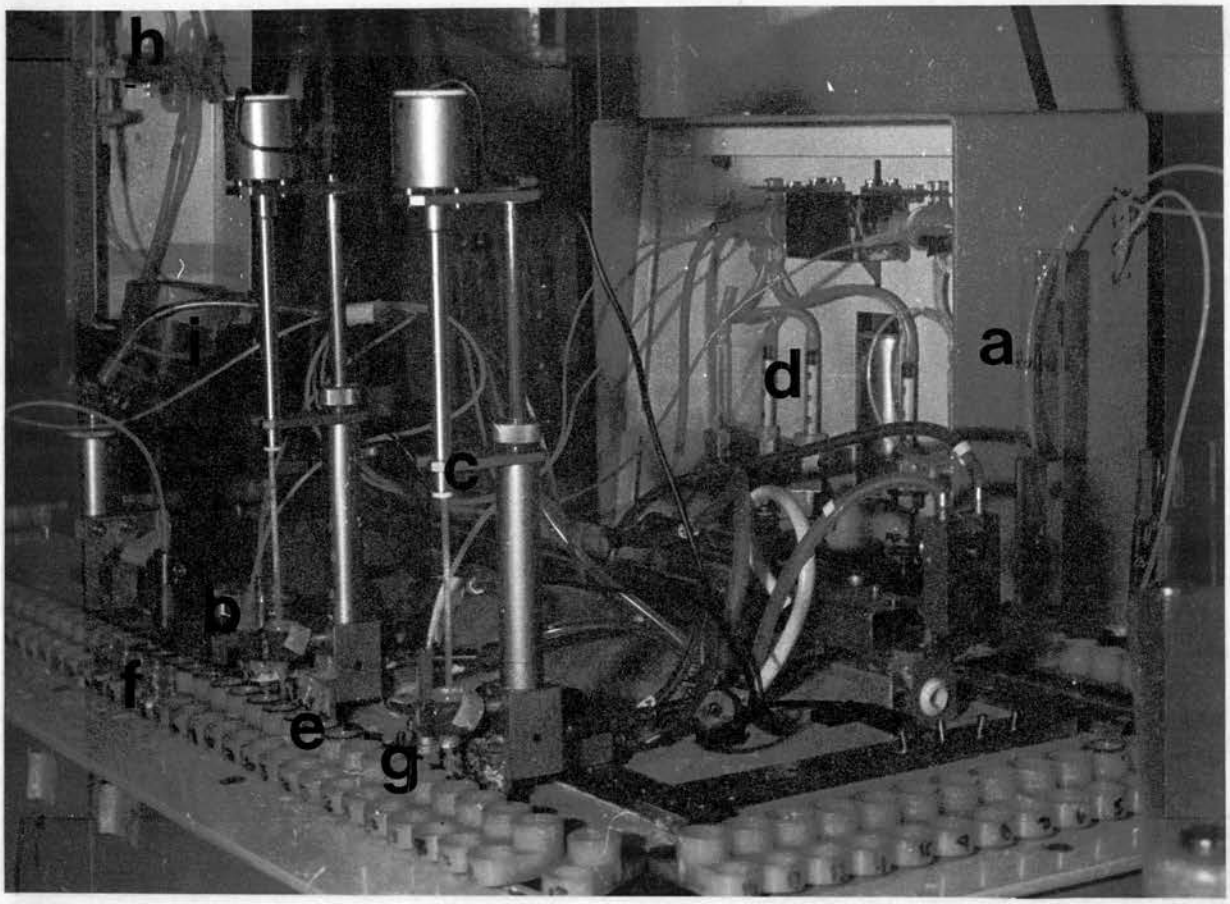
We wanted a system which would handle the samples discreetly and then scan each one. A fluorescence curve not only gives the data required for differential assay but also serves as a useful check on the performance of each individual assay (i.e. from the shape of the curve showing contamination, etc.).

A Unicam AC60 Chemical Pressing Unit was available and instead of using one of the published continuous flow methods, we decided to adapt this instrument to perform a modification of the manual technique with which we already had much experience - (in any case there was no hope of obtaining a Technicion "Auto-Analyser").

The AC60 is a discreet handling system in which a conveyor belt carries test tubes containing the samples past a series of stations which each add a reagent and then stir the mixture. One station has the added facility of being able to transfer to each individual test tube a precise amount of fluid from a plastic cup riding beside the tube. We used this to dispense standards with great precision. When all the reagents have been added, the AC 60 has a pick-up station designed to transfer the final mixture to a flow-through cell. However, we did not attempt to use this station as we did not have a flow-through cell for the Aminco Bowman Spectrophotofluorometer.

The endless track (conveyor belt) consists of 120 sample carriers which move on by one position every half a minute and thus the times between reagent additions are determined by the distances between

FIGURE 14.



UNICAM AC 60

CHEMICAL PROCESSING UNIT

Reagent delivery system:-

a Reagent container
b Dispensing station
c Stirrer
d Dispensing syringe

Track:-

e Sample tube
f Plastic cup for standard
g Lead pin

Adrenal gland perfusion system:-

h Perfusate change over multitap
i Heating jacket with inner funnel - holds Adrenal gland

the stations. The reagents are dispersed by an ingenious mechanism whereby syringes are moved pneumatically and the actual volume is determined by the travel between two rigid metal templates. (Figure 14).

Each of the 120 carriers on the track can hold a small plastic cup, a 5 ml test tube and an activating pin. Such a pin is placed beside the first sample in a batch of tubes and "switches on" the dispersing station as it passes. Similarly, a pin placed at the end of the batch turns off the stations.

Sample Collection

We used the moving track of the AC 60 as a fraction collector for the adrenal gland's effluent and thus samples were collected over thirty seconds (i.e. 1 ml).

The adrenal gland, surrounded by its heating jacket, was suspended in a small funnel over the track. A short piece of Portex tubing from the spout of the funnel was positioned so that it just touched the rims of the moving test tubes and thus prevented loss of effluent as each tube moved on. (See diagram). A funnel attached to p15
a waste pipe was fixed beneath the collection point in order to catch the effluent between collections.

Catecholamine Estimation

Once a drug infusion test was completed the batch of tubes was lined up as follows in preparation for the addition of reagents:-

(i) Lead tube - a control collection which became the faded blank.

(ii) Reagent blank - Locke's solution (1 ml) with drug which had not perfused the gland.

(iii) Standard tubes - One for adrenaline and one for noradrenaline.

(iv) Samples - Before, during and after the drug infusions.

(v) Standard tubes - (as for iii).

A metal pin is placed beside the lead tube and another in the position behind the last tube, which will later be occupied by the faded blank tube ".

Reagents

The concentrations of the reagents was altered from that used in the manual technique in order that:

(a) The final volume would be sufficient for the tubes to be "read" directly in the modified spectophotofluorometer cell.

(b) With each reagent, the volume added would be adequate to mix with the solution already in the tube.

The reagents were as follows:

	Concentration	Volume	Addition Time
Phosphate buffered Locke's Solution (i.e. adrenal gland perfusate)	-	1 ml	-
Potassium ferricyanide	0.04%	0.5 ml	0 mins
With cupric chloride	0.0004%		
Sodium sulphite	10%	0.5 ml	3 mins
With B.A.L.	0.25%		
Sodium hydroxide	8 N	0.5 ml	$3\frac{1}{2}$ mins
Acetic acid (to give final pH 5.0)	Glacial	0.4 ml	$7\frac{1}{2}$ mins

Blanks

A faded blank was most convenient as it could be incorporated easily into the reagent delivery sequence. With the arrangement of tubes described above, the first tube of a run received none of the BAL/sodium sulphite mixture while the last tube received only this mixture. Therefore, by transferring the lead tube to the end of the run after it had passed all the stations, it received its BAL/sodium sulphite after sufficient time had elapsed for its fluorescence to fade. We normally took one of the control adrenal collections for the faded blank but from time to time we used one of the collections taken during the drug infusion in case the drug released fluorescent contaminants from the gland.

Reagent blanks had 1 ml of the drug containing Locke's solution which had not perfused the adrenal gland.

Standards

Tubes containing 1 ml of Locke's solution were prepared and incorporated in the above described sequence alongside a small plastic cup containing catecholamine standard solution (10 µg/ml). The AC 60 transferred a precise quantity of standard (200 mg) from the plastic cup to the test tube.

We usually had two pairs of standards with each batch of samples - one pair before and one after the samples.

Reading the Fluorescence

The AC 60 holds pyrex test tubes (12 x 75 mm) and these were found to have no more absorbance than quartz in the range of the spectrum covering the excitation and emission wavelengths for the differential assay.

It was possible to modify the Aminco-Bowman spectrophotofluorometer (using a plastic pill container) so that it would hold the round pyrex tubes. (We took great care to rinse and wipe the outside of the tubes before insertion into the Aminco-Bowman).

Thus we eliminated the potentially expensive and very time consuming process of filling and rinsing quartz cuvettes.

Flaws in the pyrex tubes could be detected by rotating them while in the fluorimeter and seeing if the fluorimeter reading was affected. Any such tube would have been discarded but no flaws were found. In addition, the tubes were found to be of constant diameter - judged by their precise fit into the conveyor belt holes and the modified cell on the Aminco-Bowman.

The manual transfer of samples to the Aminco-Bowman was so simplified that it would have been pointless - (for our requirements) to have attempted to use the automatic transfer system. The time required to perform the fluorescence scan was now the limiting factor in the assay.

SOME COMMENTS ON THE ISOLATED ADRENAL GLAND PERFUSION SYSTEM

The retrograde perfusion of the adrenal gland via the adreno-lumbar vein was necessary because the gland is supplied by a multitude of arteries while there is, usually only one vein. Thus in order to perfuse the gland through its arterial supply we would have had to perfuse the adrenal glands, in situ, via the aorta. It was far easier to cannulate the adreno-lumbar vein and avoid the various practical problems involved in the routine use of a whole animal experiment. Also, retrograde perfusions avoid the influence of any adrenocortical mediated effects on the medulla.

Phosphate buffered Locke's solution was well established as an adrenal perfusate and had been employed by both M. Vogt and W.W. Douglas. Vogt (1965) considered that when using this perfusion technique the "in vitro" responses to bradykinin and angiotension were much less than are found "in vivo" but considered that the receptors sensitive to acetyl choline and potassium ions survived well. Nahas (1970) recommended using diluted homologous blood for the perfusion of the isolated dog adrenal gland as his electron microscope studies had shown that this medium preserved the fine structure of the gland. However, the use of the latter perfusate, or even a plasma substitute, would have necessitated an extraction procedure prior to the fluorimetric assay.

The time taken to estimate samples would have been considerably lengthened with a consequent reduction in the number of experiments performed and loss of the "on-line" advantages of our automated assay. N.B. An on-line assay could have been set up using a dialysis exchanger coupled to a continuous flow analysis system (e.g. Technicion "Auto-

"Auto-Analyser") but this combined with the more sophisticated circulation/ gland perfusion needed to justify the expensive perfusate, was economically unfeasible. (G.G. Nahas works for the U.S. Army).

All adrenal gland perfusion systems can be criticised on the grounds of being "artificial" or "unphysiological". We wanted to use the results for our isolated gland experiments to back up our "whole animal" research and we were well aware that the artificial perfusion technique has its limitations.

Thus we chose our technique on the grounds of its simplicity and capacity to perform on-line estimations which enabled us to do a great many more estimations than would otherwise have been possible.

Bülbring, Burn & De Elio (1949) report weighing their glands at the end of the perfusion in order to estimate the loss of catecholamines. Our glands tend to swell during perfusion and were often several hundred milligrammes heavier by the end of the experiment.

We also determined whether our glands were hypoxic during their perfusion with oxygenated Locke's solution. This was performed by collecting the effluent under liquid paraffin. (i.e. immersing the gland in a small pot containing liquid paraffin) and measuring the PO_2 which was usually over 70 torr.

At the end of some of our experiments, we perfused the gland with Locke's solution containing either Gentian Violet or Trypan Blue. Following formaldehyde fixation, paraffin wax sections (some stained with haematoxylin and eosin) were made by the Department of Anatomy. We found that the dye was always distributed evenly throughout the whole of the adrenal medulla. The opinion of several experienced histologists (eg. Edward Duvall - Sir William Dunn School of Pathology, Oxford) was that the cells in the adrenal medulla appeared to have survived the perfusion very well.

RESULTS

I have displayed our results in graphical form. The details of each individual experiment are recorded in the Tables at the end of this thesis. P290

INVESTIGATION OF SELECTIVE ADRENAL CATECHOLAMINE RELEASE

I have described below how the results are grouped for the two species and in each case I have given the mean ± S.E.M.

The noradrenaline, adrenaline and total catecholamine outputs are shown in ng/kg.min. for the whole animal and in ng/min. for the isolated gland experiments.

The percentage of noradrenaline in the total is displayed in two forms:-

(a) Mean ± S.E.M. of the individual estimations of the noradrenaline percentage in the group. P223-4

(b) A value calculated from the mean noradrenaline and total catecholamine output for the group.

DOGS - WHOLE ANIMAL EXPERIMENTS

The results displayed in Fig.15 are taken from all our dog experiments and show the mean noradrenaline and adrenaline outputs during the baroreceptor and chemoreceptor tests with their combined resting outputs. We excluded those tests performed after the administration of a drug (i.e. Synacthen, cycloheximide, hexamethonium, and atropine). However, those tests performed after guanethidine administration were included and we did not observe any effect of this drug on adrenal catecholamine output (resting or reflex release) in our experiments DR1-3. P17 P1

CONTROLS

Baroreceptor and chemoreceptor resting outputs are combined.

*Control Output (n = 83)

Noradrenaline = 2.5 ± 0.3 ng/kg.min.

Adrenaline = 11.5 ± 1.1 ng/kg.min.

Total Catecholamine = 14.1 ± 1.3 ng/kg.min.

NA % (a) 18.9 ± 1.0 (b) 18

BARORECEPTOR TESTS

These were performed by lowering carotid sinus pressure from 142 ± 2 to 80 ± 1 mmHg while the carotid perfusion blood parameters were:-

PO_2 = 182 ± 22 torr

PCO_2 = 41 ± 2 torr

pH = 7.33 ± 0.01

*Incremental release during Baroreceptor stimulation (n = 36)

Noradrenaline = 4.4 ± 0.8 ng/kg.min.

Adrenaline = 18.7 ± 3.9 ng/kg.min.

Total Catecholamine = 23.0 ± 4.7 ng/kg.min.

NA % (a) 18.7 ± 1.7 (b) 19

CHEMORECEPTOR TESTS

The releases that were measured during the first period of prolonged chemoreceptor stimulation have been included.

The carotid sinus pressure was maintained at 142 ± 2 mmHg and during the test the blood perfusing the carotid bifurcation was changed from:-

PO_2	=	84 ± 7 torr	to	PO_2	= 34 ± 3 torr
PCO_2	=	41 ± 2 torr		PCO_2	= 58 ± 5 torr
pH	=	7.34 ± 0.07		pH	= 7.27 ± 0.03
		(Control)			(Test)

*Incremental release during Chemoreceptor stimulation (n = 17)

Noradrenaline = 2.1 ± 0.5 ng/kg.min.

Adrenaline = 11.0 ± 3.5 ng/kg.min.

Total Catecholamine = 13.1 ± 3.9 ng/kg.min.

NA % (a) 20.0 ± 3.0 (b) 16

Incremental release during Chemoreceptor stimulation, including those tests after Cycloheximde infusion (n = 23)

Noradrenaline = 2.4 ± 0.4 ng/kg.min.

Adrenaline = 12.8 ± 2.7 ng/kg.min.

Total Catecholamine = 15.2 ± 3.0 ng/kg.min.

NA % (a) 18.4 ± 2.4 (b) 16

ELECTRICAL STIMULATION

P100

Noradrenaline = 37.8 ± 10.6 ng/kg.min.

Adrenaline = 130.4 ± 35.3 ng/kg.min.

Total Catecholamine = 168.2 ± 45.7 ng/kg.min.

NA % (a) 23.3 ± 2.8 (b) 23

*Used for Figure 15.

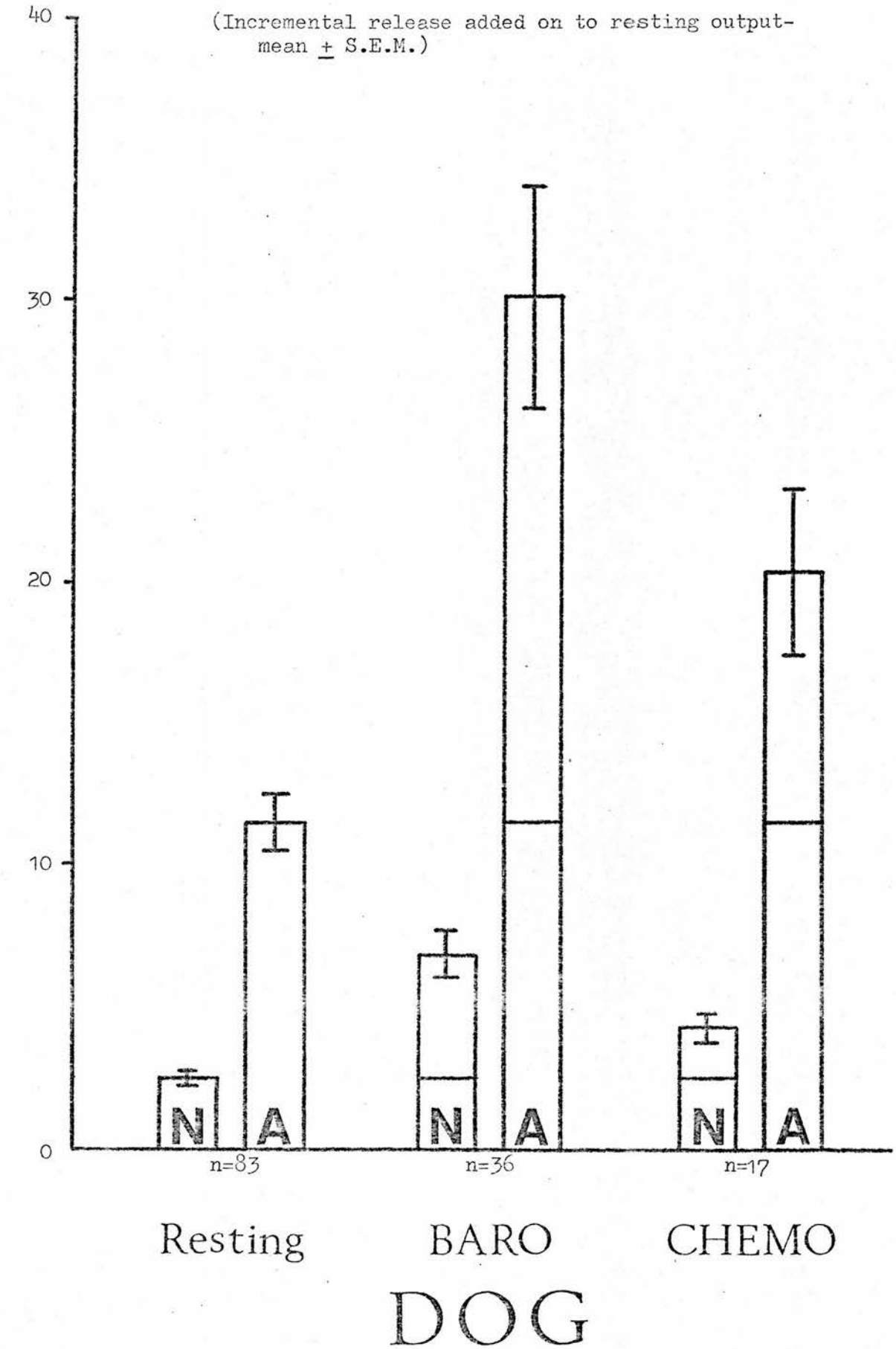
FIGURE 15.

WHOLE ANIMAL EXPERIMENTS

NA & A OUTPUTS at REST

and during BARORECEPTOR & CHEMORECEPTOR tests

(Incremental release added on to resting output- mean ± S.E.M.)



DOGS - ISOLATED GLAND EXPERIMENTS

The results in the Tables are expressed in ng/min. as control output and incremental release for each individual drug infusion. The control output is the average of the three resting collections. (The fourth control tube was used in the assay as the faded blank).

The incremental release was calculated by subtracting the mean control output from the peak output per minute obtained during the drug infusion.

For Fig. 16 which compares the proportions of adrenaline and noradrenaline released by the glands during stimulation with methacholine and hpp TMA, we have used the incremental releases from all the isolated gland experiments. The controls are the combined mean resting collections from all the experiments. p.173

*CONTROLS (n =46)

Noradrenaline = 81 ± 11 ng/min.

Adrenaline = 307 ± 39 ng/min.

Total Catecholamine = 387 ± 49 ng/min.

NA % (a) 20.7 ± 1.0 (b) 21

INCREMENTAL RELEASE WITH hpp TMA (n = 25)

Dose = 10^{-8} - 10^{-5} Molar

In order to have incremental releases of equivalent size with the two drugs when drawing Figure 16, we excluded the releases over 3000 ng/min. p 339

DOGS (cont)

*Excluding releases 3000 ng/min. (n = 18)

Noradrenaline = 183 ± 41 ng/min.

Adrenaline = 574 ± 144 ng/min.

Total Catecholamine = 757 ± 180 ng/min.

NA % (a) 25.4 ± 2.0 (b) 24

Including releases 3000 ng/min. (n = 25)

Noradrenaline = 505 ± 120 ng/min.

Adrenaline =1517 ± 346 ng/min.

Total Catecholine =2021 ± 438 ng/min.

NA % (a) 25.4 ± 1.6 (b) 25

*INCREMENTAL RELEASES WITH METHACHOLINE (n = 21)

Dose = 10^{-8} - 10^{-6} Molar

Noradrenaline = 134 ± 30 ng/min.

Adrenaline = 364 ± 74 ng/min.

Total Catecholamine = 498 ±101 ng/min.

NA % (a) 24.5 ± 1.7 (b) 27

*Used in Figure 16.

Response to hpp TMA abolished by Hexamethonium (3×10^{-4} M)

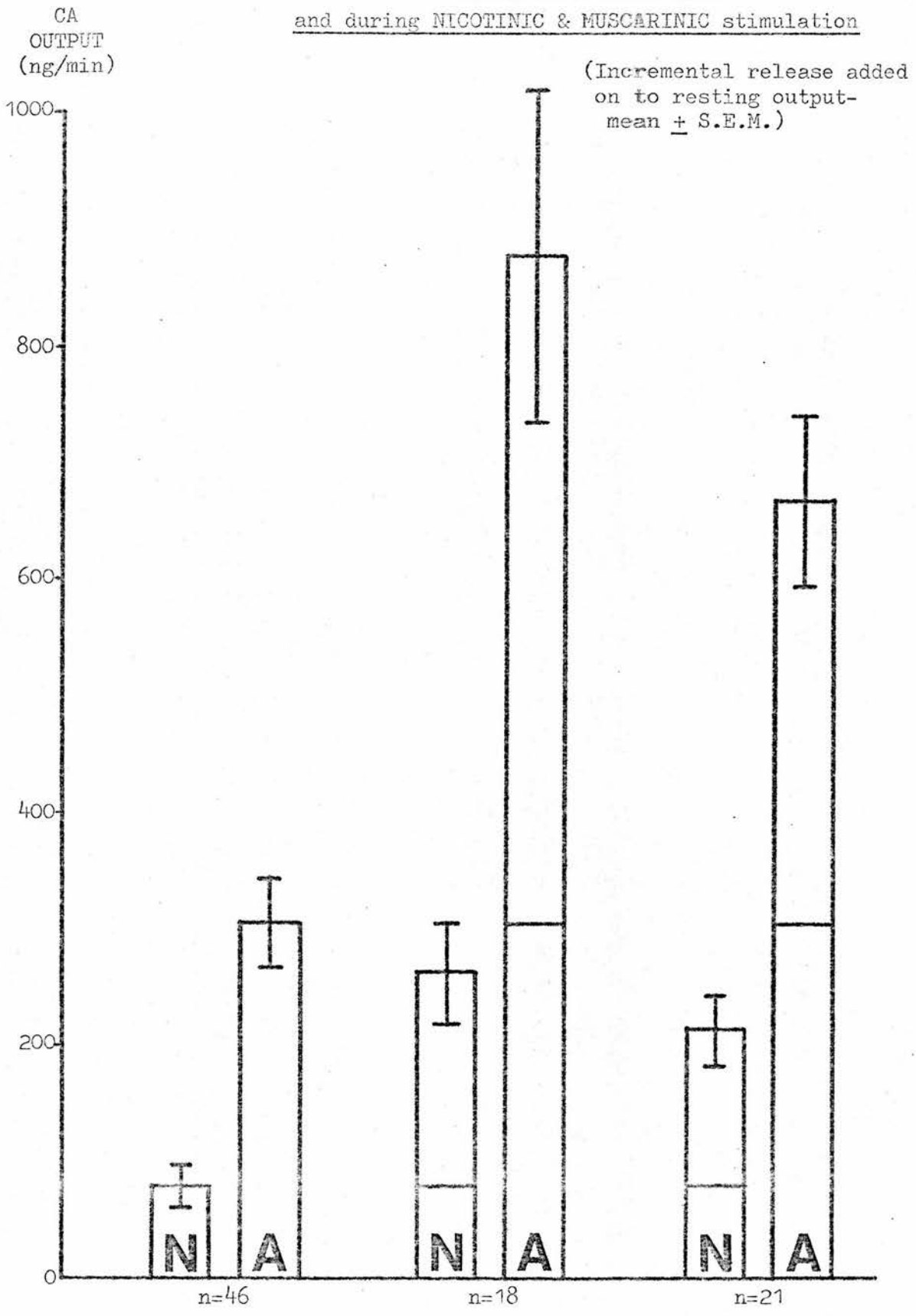
Response to Methacholine abolished by Atropine (3×10^{-5} M)

FIGURE 16.

ISOLATED ADRENAL GLAND EXPERIMENTS

NA & A OUTPUTS at REST

and during NICOTINIC & MUSCARINIC stimulation



DOG

CATS - WHOLE ANIMAL EXPERIMENTS

Only the tests and controls collected before the administration of hexamethonium and/or atropine are included. P176

CONTROLS

Baroreceptor and Chemoreceptor test controls are considered together.

*Control output (n = 30)

Noradrenaline = 3.2 ± 0.6 ng/kg.min.

Adrenaline = 3.0 ± 0.4 ng/kg.min.

Total Catecholamine = 6.1 ± 0.8 ng/kg.min.

NA % (a) 45.8 ± 4.0 (b) 52

BARORECEPTOR TESTS

These were performed by lowering carotid sinus pressure from 150 ± 2 to 84 ± 4 mmHg while the carotid perfusion blood parameters were:-

PO_2 = 208 ± 26 torr
PCO_2 = 27 ± 1 torr
pH = 7.36 ± 0.04

*Incremental release during Baroreceptor stimulation (n = 14)

Noradrenaline = 3.3 ± 0.7 ng/kg.min.

Adrenaline = 1.5 ± 0.4 ng/kg.min.

Total Catecholamine = 4.8 ± 0.8 ng/kg.min.

NA % (a) 73.5 ± 5.9 (b) 70

CATS CHEMORECEPTOR TESTS

The carotid sinus pressure was maintained at 149 ± 3 mmHg and during the test the carotid perfusion blood was changed from:-

PO_2 = 262 ± 47 torr	to	PO_2 = 34 ± 14 torr
PCO_2 = 29 ± 2 torr		PCO_2 = 69 ± 11 torr
pH = 7.38 ± 0.03 (Control)		pH = 6.79 ± 0.05 (Test)

*Incremental release during Chemoreceptor stimulation (n = 10)

Noradrenaline	= 5.2 ± 3.2 ng/kg.min.
Adrenaline	= 12.8 ± 6.1 ng/kg.min.
Total Catecholamine	= 18.0 ± 9.1 ng/kg.min.
NA % (a) 16.3 ± 5.1	(b) 29

*Used in Figure 17.

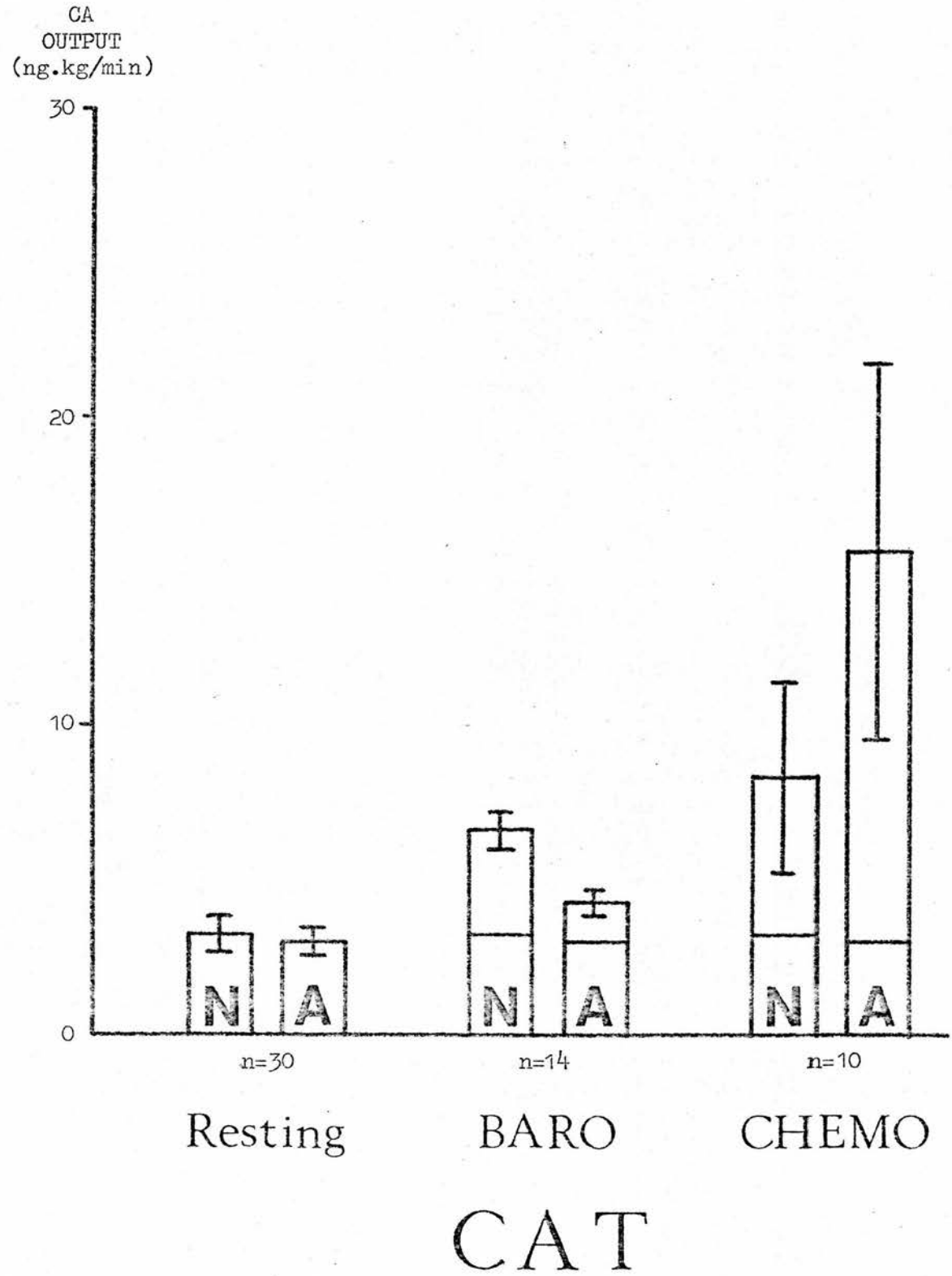
FIGURE 17.

WHOLE ANIMAL EXPERIMENTS

NA & A OUTPUTS at REST

and during BARORECEPTOR & CHEMORECEPTOR tests

(Incremental release added on to resting output- mean ± S.E.M.)



CATS - ISOLATED GLAND EXPERIMENTS

The results are recorded in the Tables and calculated in the same way as has been described for dogs. Figure 18 is based on the incremental releases from all the isolated gland experiments, excluding those tests performed after addition of Hexamethonium or Atropine to the perfusing fluid. The controls are the combined resting collections form all the experiments, again excluding those in which a blocking drug is present. P178

*Control outputs (n = 52)

Noradrenaline = 27 ± 3 ng/min.

Adrenaline = 56 ± 6 ng/min.

Total Catecholamine = 83 ± 9 ng/min.

NA % (a) 37.0 ± 1.7 (b) 32

*Incremental releases with hpp TMA (n = 27)

Noradrenaline = 261 ± 64 ng/min.

Adrenaline = 168 ± 26 ng/min.

Total Catecholine = 429 ± 87 ng/min.

NA % (a) 56.5 ± 2.5 (b) 61

*Incremental releases with Methacholine (n = 25)

Dose = 5×10^{-6} - 10^{-4} Molar

Noradrenaline = 99 ± 11 ng/min.

Adrenaline = 189 ± 19 ng/min.

Total Catecholamine = 288 ± 28 ng/min.

NA % (a) 34.4 ± 1.5 (b) 34

Response to hpp TMA abolished by Hexamethonium (3×10^{-4} M)

Response to Methacholine abolished by Atropine (3×10^{-5} M)

*Used in Figure 18.

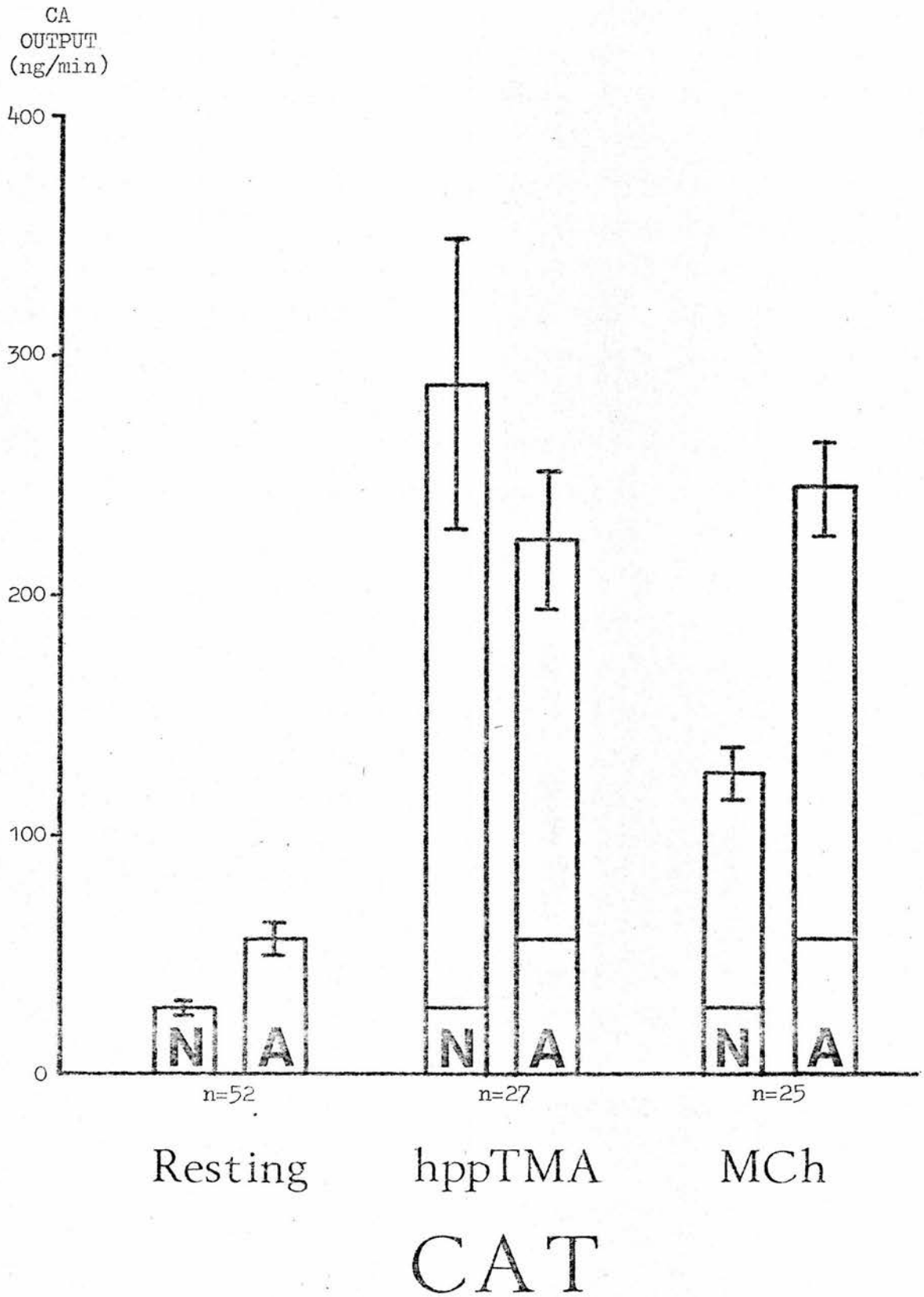
FIGURE 18.

ISOLATED ADRENAL GLAND EXPERIMENTS

NA & A OUTPUTS at REST

and during NICOTINIC & MUSCARINIC stimulation

(Incremental release added on to resting output-
mean ± S.E.M.)



Dose-Response Relationship

We had used the automated assay for all the isolated cat adrenal gland work and were able to draw a log dose-response curve for both hpp-TMA and methacholine. The results with the two drugs are shown in Figure 19 and for each drug I have drawn:-

(a) A regression line using all our results (by the method of least squares)

(b) The mean $\pm$ S.E.M. for four of the doses

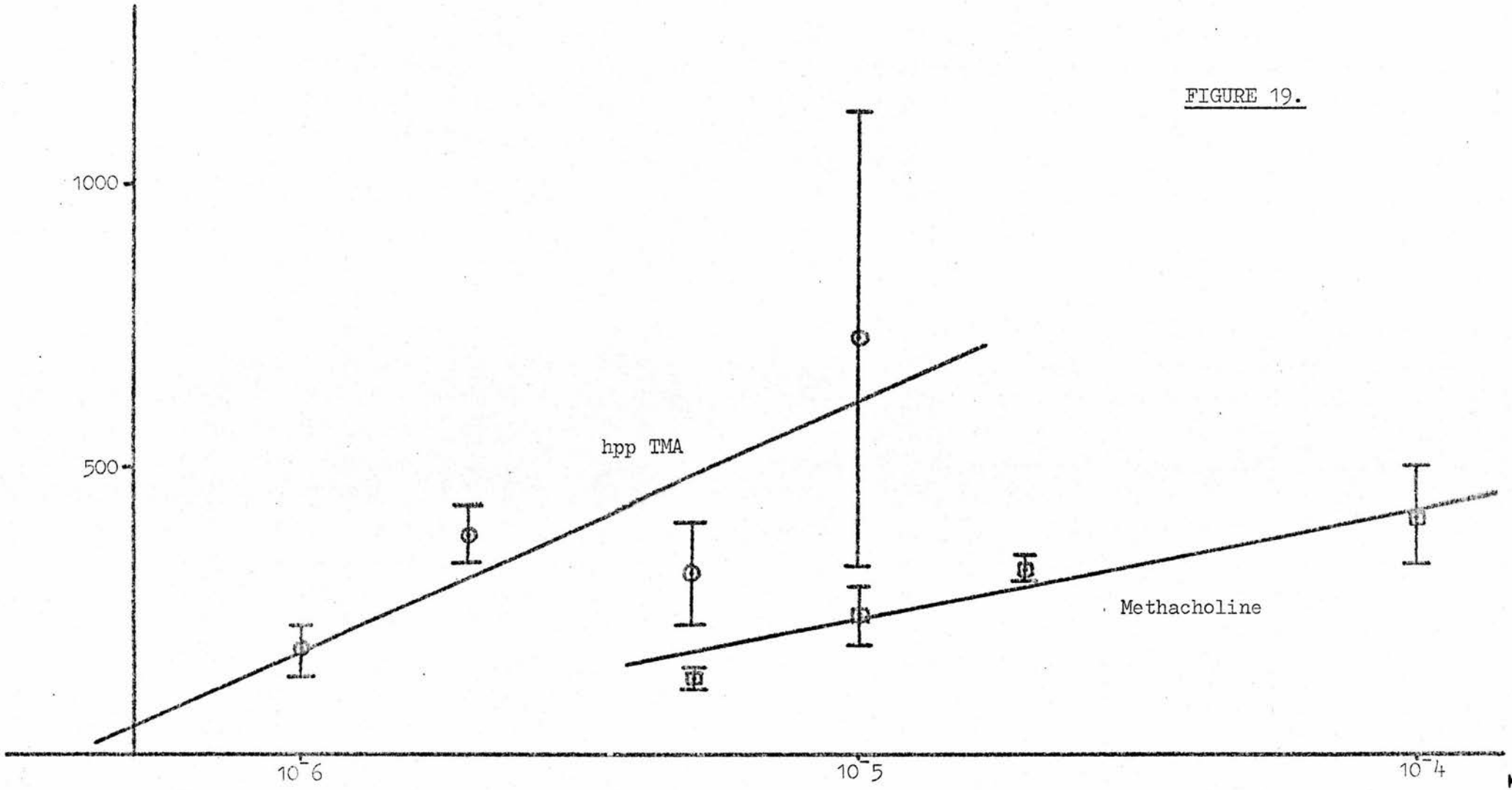
CATS

ISOLATED GLANDS

LOG DOSE-RESPONSE CURVE for hpp TMA & METHACHOLINE

For each drug:-
a) Regression line plotted by method of least squares.
b) Mean ± S.E.M. for 4 doses.

FIGURE 19.



THE EFFECTS OF MUSCARINIC AND NICOTINIC ANTAGONISTS IN CATS

1. The Actions of Hexamethonium and Atropine on Catecholamine Release from Isolated Cat Glands

(a) Hexamethonium

In glands C4 and C6 Hexamethonium (3×10^{-4} M) was added to the perfusing Locke's solution.

It was found (see tables of results) to completely abolish the release with hpp TMA while release still occurred with Methacholine and Carbachol. p 343-7

(b) Atropine

In gland C7 Atropine (3×10^{-5} M) was added to the perfusing fluid. This blocked release with Methacholine, while release still occurred with hpp TMA and to a lesser extent with carbachol.

2. The Actions of Hexamethonium and Atropine on the Baroreceptor and Chemoreceptor Reflexes in Cats

(a) Hexamethonium

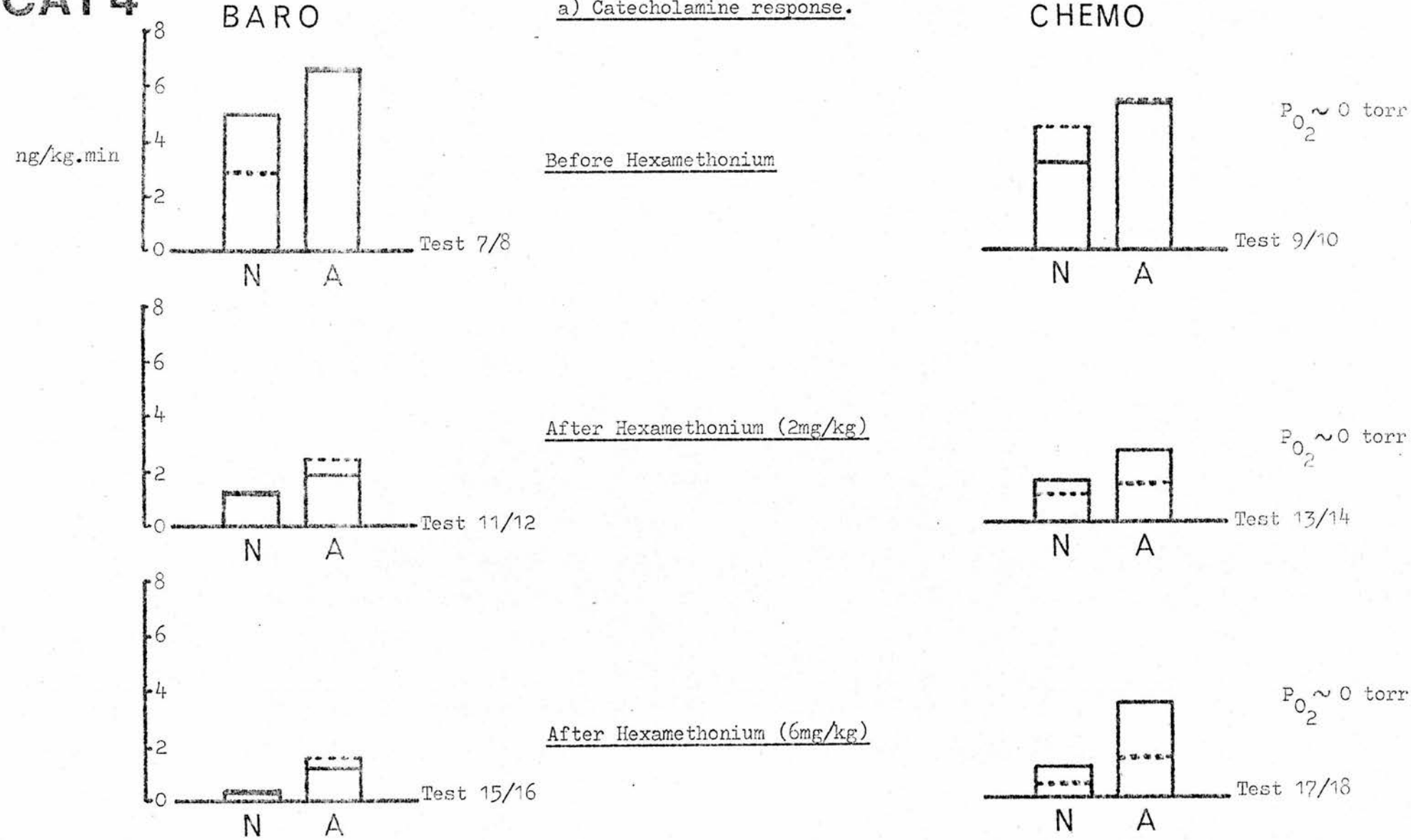
In Cat 4 and 5 we found that the blood pressure and adrenal medullary responses to chemoreceptor stimulation were resistant to doses of hexamethonium (2 - 6 mg /kg) which abolished both these components of the baroreceptor reflex. These results are shown in Figures 20-23.

In both these experiments, atropine administration after hexamethonium resulted in a rapid fall in blood pressure which was followed by death.

CAT 4

Effect of Hexamethonium on Baroreceptor and Chemoreceptor reflexes.

a) Catecholamine response.



Test output superimposed on control (----) output.

FIGURE 20.

FIGURE 21.

Baroreceptor and Chemoreceptor reflexes in CAT 4 which received Hexamethonium.
b) Systemic blood pressure response

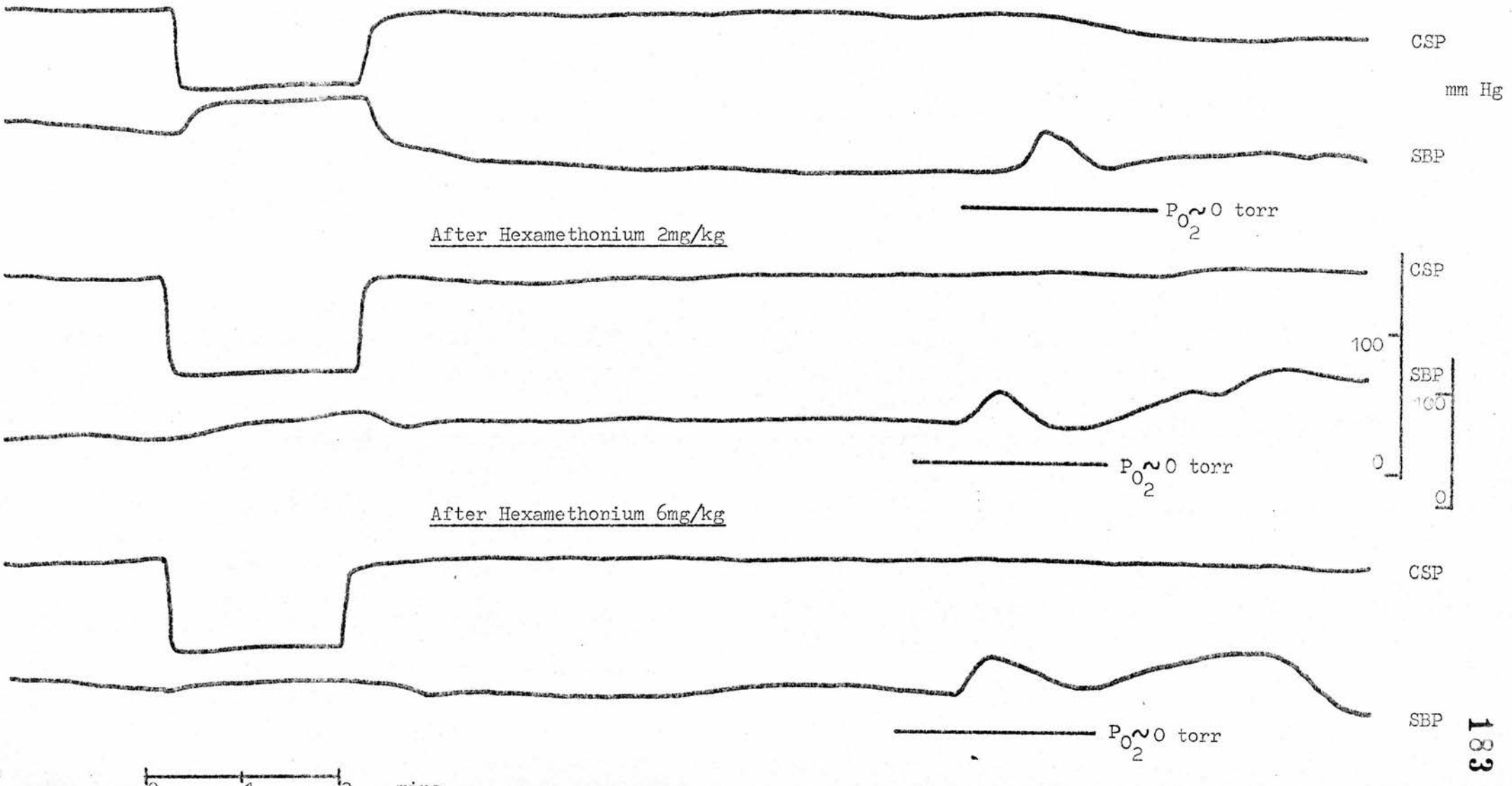
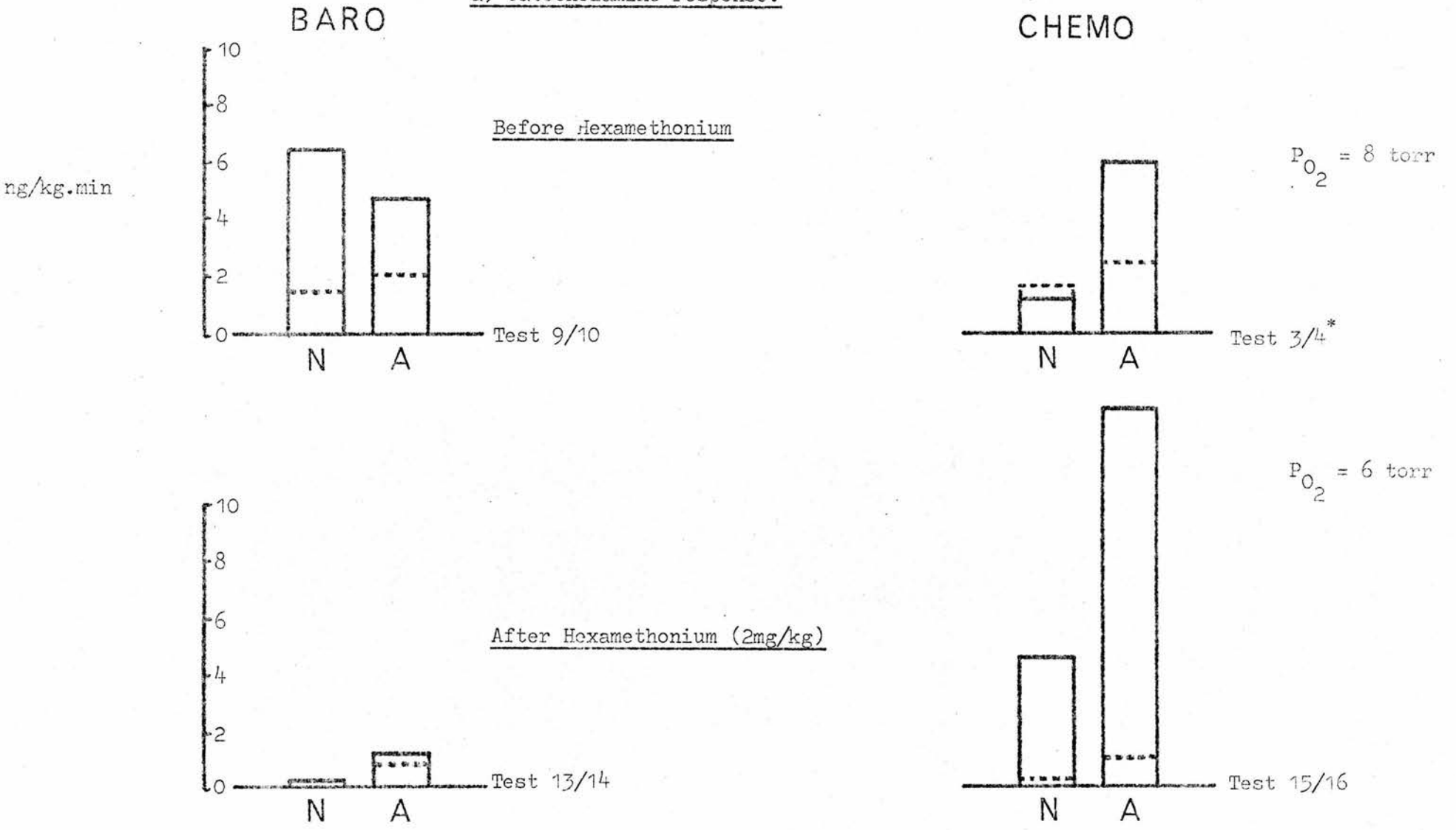


FIGURE 22.

CAT 5

Effect of Hexamethonium on Baroreceptor and Chemoreceptor reflexes.

a) Catecholamine response.



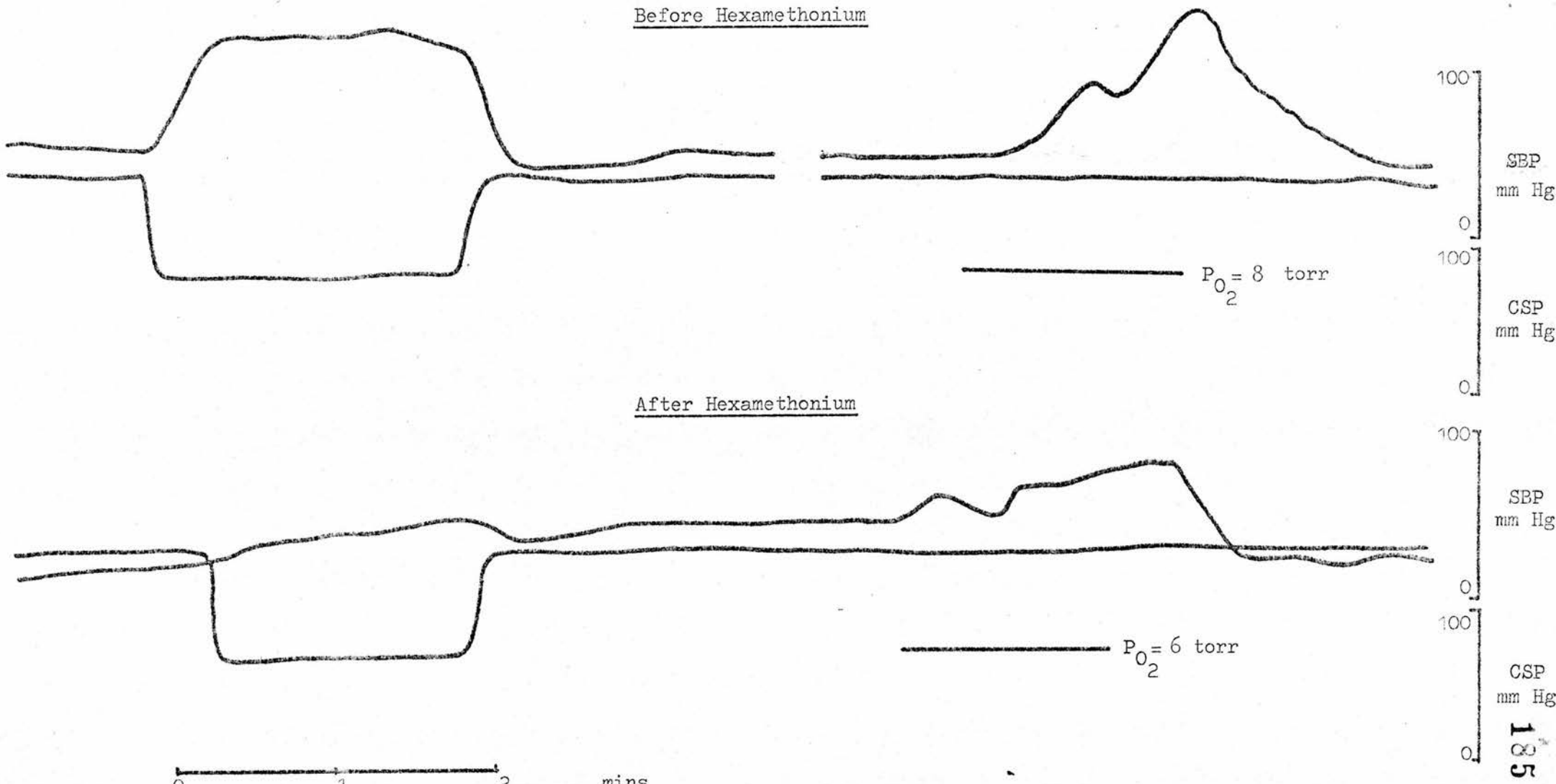
Test output superimposed on control (----) output

*Chosen because comparable P_{O_2} to 15/16

FIGURE 23.

Baroreceptor and Chemoreceptor reflexes in CAT 5 which received Hexamethonium (2mg/kg)

b) Systemic blood pressure response



(b) Atropine

Atropine alone did not abolish the adrenal or the blood pressure components of either reflex. Furthermore, in Cat 6 (see Figure 24) an adrenal medullary response to chemoreceptor stimulation was only seen after atropine administration.

The phenomenon of selective release was still seen after atropine administration. In Cat 6, following the first dose (50 µg /kg), the incremental releases contained 79% NA with the baroreceptor and 58% with the chemoreceptor tests. (NB Nearly equivalent sized releases of adrenaline). Similarly after the second dose (1 mg /kg), the baroreceptor reflex released 79% NA while the chemoreceptor reflex released 57% NA and again the adrenaline releases were almost equal. p187

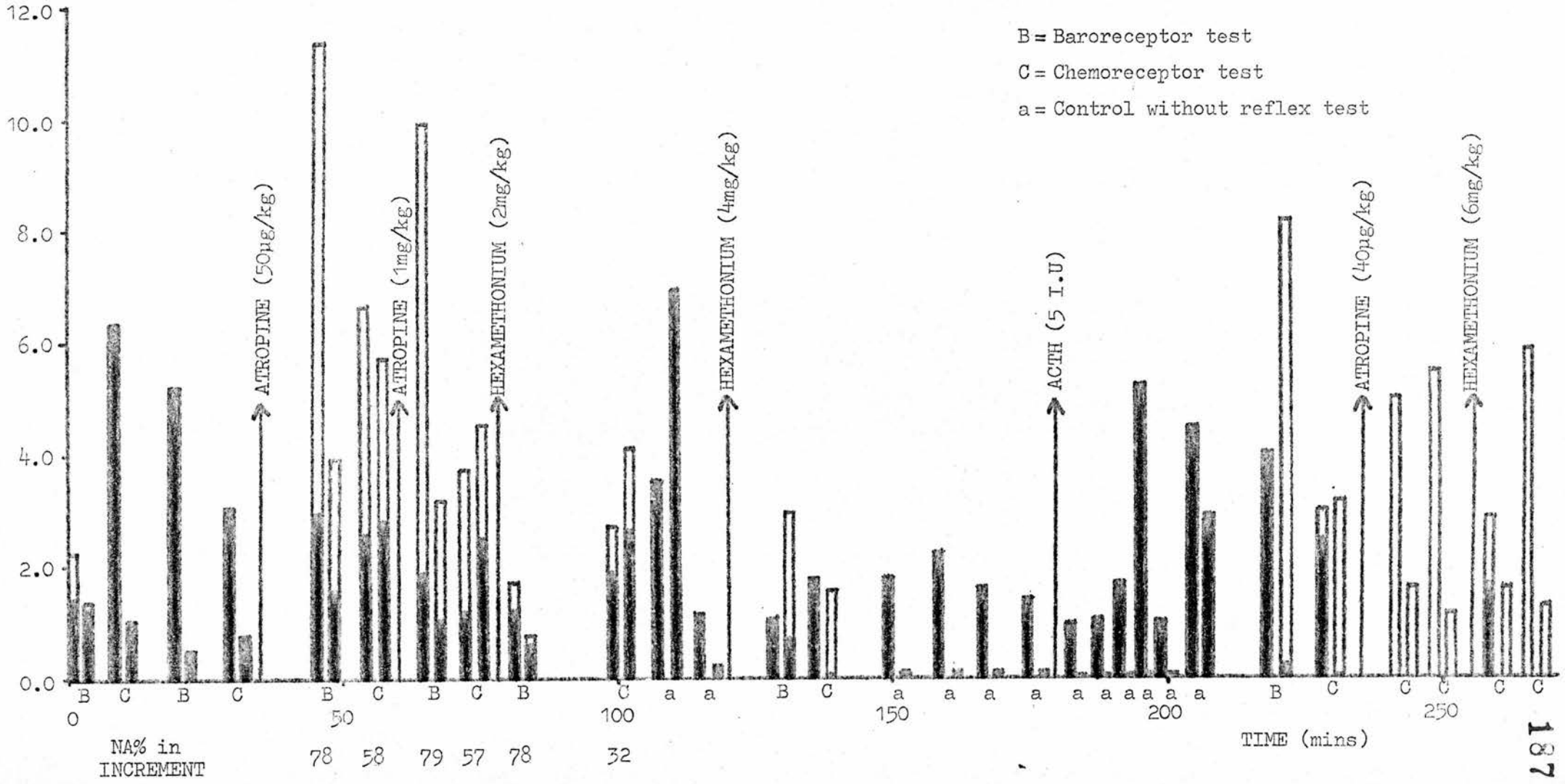
Hexamethonium (2 mg /kg) then almost abolished the adrenal medullary component of the baroreceptor reflex while the chemoreceptor release was halved and now contained 32% NA.

We then investigated the effect of synacthen (1 IU/kg) on the resting secretion. At the end of the experiment we obtained releases with chemoreceptor stimulation which contained over 50% NA.

In Cat 7 although there was no adrenal medullary response to chemoreceptor stimulation after atropine (100 µg /kg), an equivalent stimulus gave a release consisting solely of adrenaline following the administration of another dose (1 mg /kg). (See tables of results). p324

FIGURE 24.

CAT 6



HUMORAL CONTROL OF ADRENAL CATECHOLAMINE OUTPUT

In brief our results show that:-

In Whole Animals

1. The release of catecholamines provoked by hypoxic carotid chemoreceptor stimulation outlasts the stimulus and often continues to rise after its cessation. (DC1 - DC8)

2. This delayed response is not abolished by denervation of the adrenal medulla. (DC1)

3. Intravenous corticotrophin (Synacthen) produces a release of catecholamines of similar magnitude and time course which also is not abolished by denervation of the adrenal gland. (NB Other workers have shown that carotid chemoreceptor stimulation activates the anterior pituitary-adrenocortical axis).

4. The delayed release of catecholamines in response to both synacthen and to chemoreceptor stimulation is inhibited by cycloheximide, which is known to block the release of adrenal steroids.
(NB We used the reflex release from baroreceptor tests as an index that in our experiments cycloheximide was not blocking neuronally mediated catecholamine release.

In Isolated Locke-Perfused Adrenal Glands

5. The order of concentration of corticosteroids one would expect in the adrenal gland during stress releases adrenal catecholamines.

I have expressed in pictorial form the results of the individual experiments done in this investigation as I consider this to be clearer than a verbal description. (See Figures 26-35). p192 - 202

The details of each experiment can be found in the tables of results. The experiments are described in the next section.

DA 1-3

These show the increase in resting output and potentiation of the baroreceptor reflexes by Synacthen (1 IU /kg). (Figures 26-28)

DN 3

This shows that Synacthen (1 IU /kg) increases the catecholamine output from a denervated adrenal gland. We stimulated the peripheral end of the greater splanchnic nerve but did not always obtain a release.

This experiment demonstrated that Synacthen was not acting by a neurally mediated mechanism e.g. potentiating transmission in the CNS. (NB DN 3 is plotted as one of the six curves in Figure 36). p 204

DC 1

A dog, with the left adrenal gland denervated was subjected to a twenty minute period of chemoreceptor stimulation (PO_2 = 32 torr) and this was followed by a three fold increase in catecholamine output which rose to a peak thirty minutes after the cessation of the chemoreceptor stimulus. The denervation was confirmed by the abolition of the immediate reflex responses to baroreceptor (or chemoreceptor) stimulation. (Figure 29). p196

DC 8

The dog had its right adrenal gland, (i.e. gland not used for blood collection) denervated and was subjected to a ten minute period of chemoreceptor stimulation (PO_2 = 22 torr).

A massive reflex release was seen in response to the chemoreceptor stimulus and the resting output was raised by five fold, ten minutes after the end of the stimulus. (Figure 29). This experiment was to exclude the possibility that circulating catecholamines released from the right adrenal gland by chemoreceptor stimulation were activating the anterior pituitary-adrenocortical axis.

DC 2-7

The protocol is detailed in the section on methods. The magnitude of the maintained release after the end of the first chemoreceptor stimulus appears to be dependent on the degree of hypoxia achieved. (See Figure 37). After the administration of cycloheximide a more severe hypoxic chemoreceptor stimulus did not result in a maintained catecholamine release. (Figures 30-34).

p105
p206
p197 - 201

We used the response to the baroreceptor reflex as an index of adreno-medullary secretory capacity before and after cycloheximide. There is no evidence that the immediate response to baroreceptor or chemoreceptor stimulation was impaired by cycloheximide.

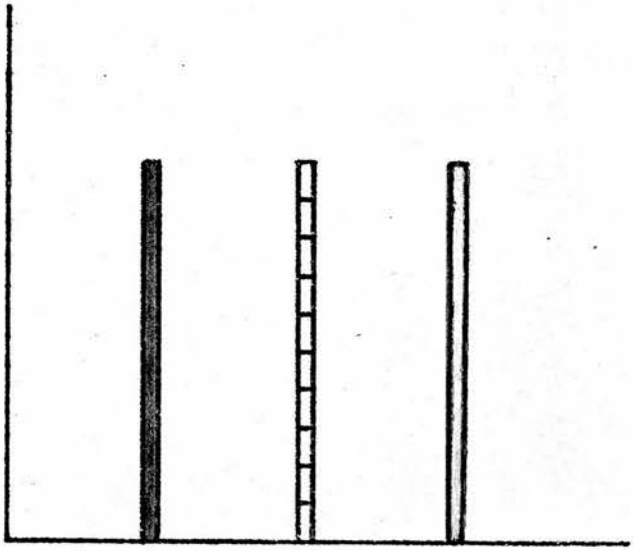
At the end of experiments DC 5-7 Synacthen (1 IU./kg) was given and although a response was always detected, it was very much reduced compared to those experiments without cycloheximide.

Experiments DC 2-7 have been combined in Figure 35.

p202

KEY

GRAPHS:- DA1-3
DC1-8

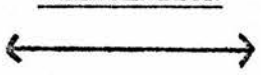


CA OUTPUTS (ng/kg.min)

NA PERCENTAGES

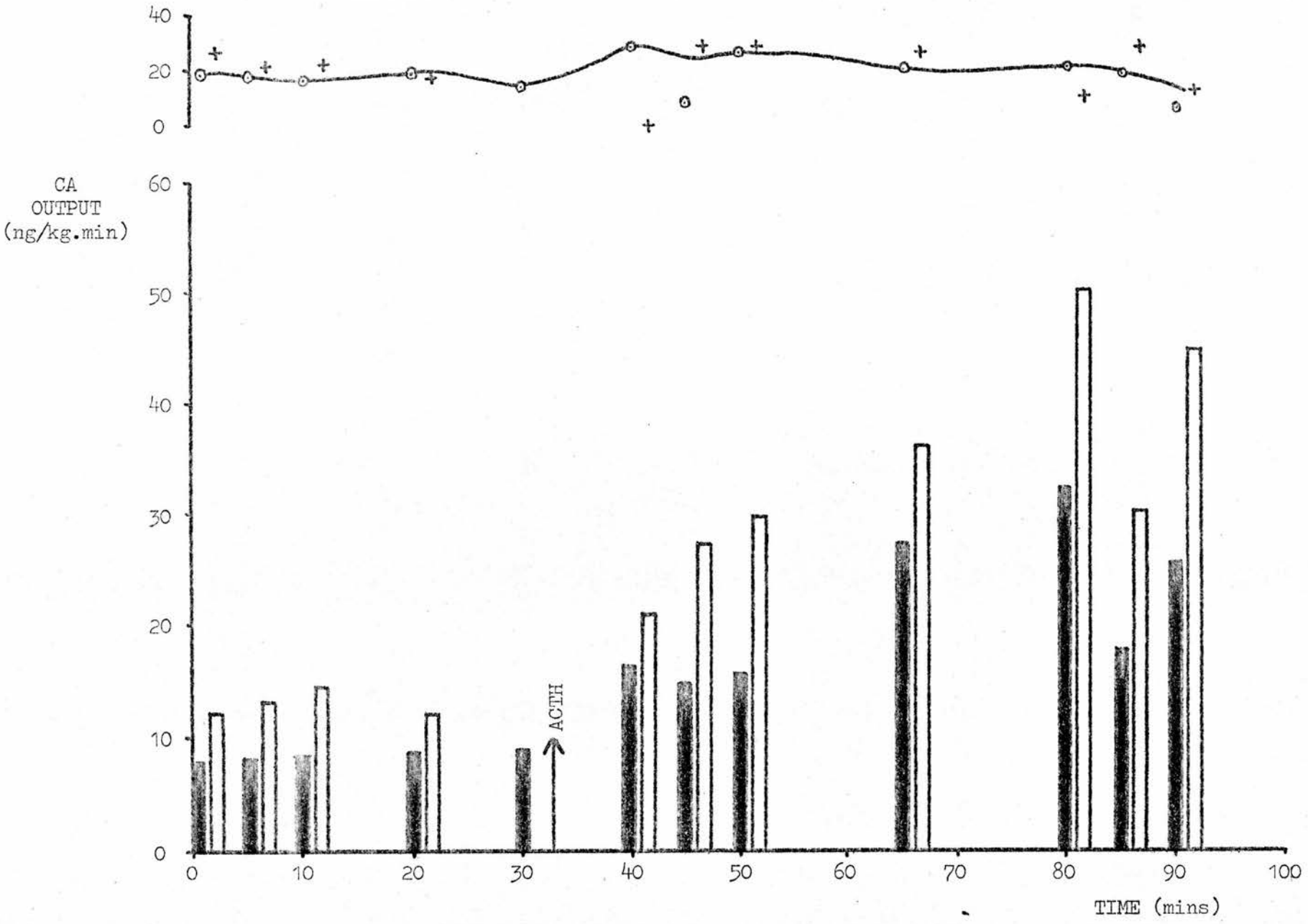
⊙ RESTING OUTPUT
✕ CHEMORECEPTOR OUTPUT
✚ BARORECEPTOR OUTPUT

PROLONGED CHEMORECEPTOR STIMULATION



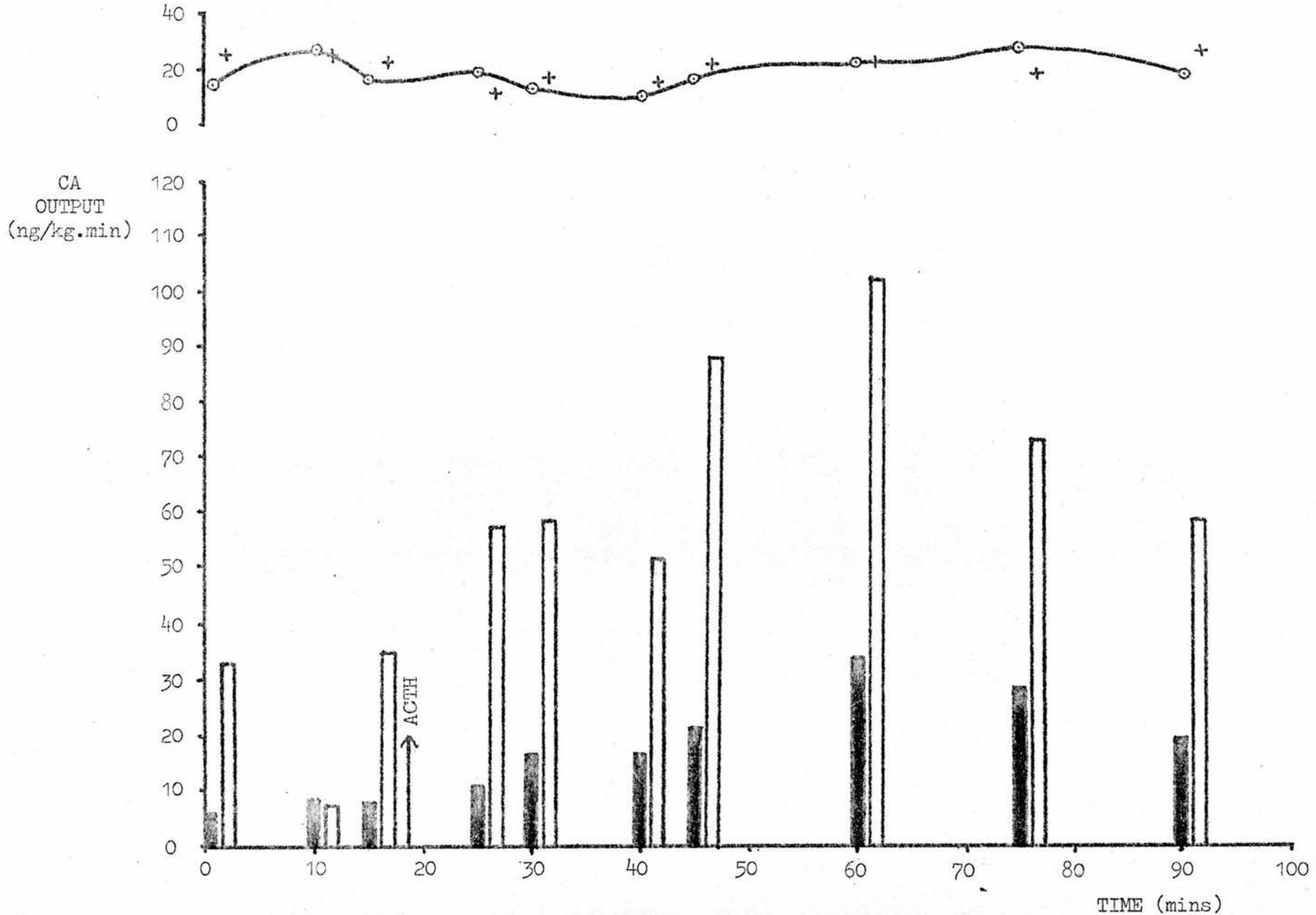
DA1

FIGURE 26.



DA2

FIGURE 27.



DA3

FIGURE 28.

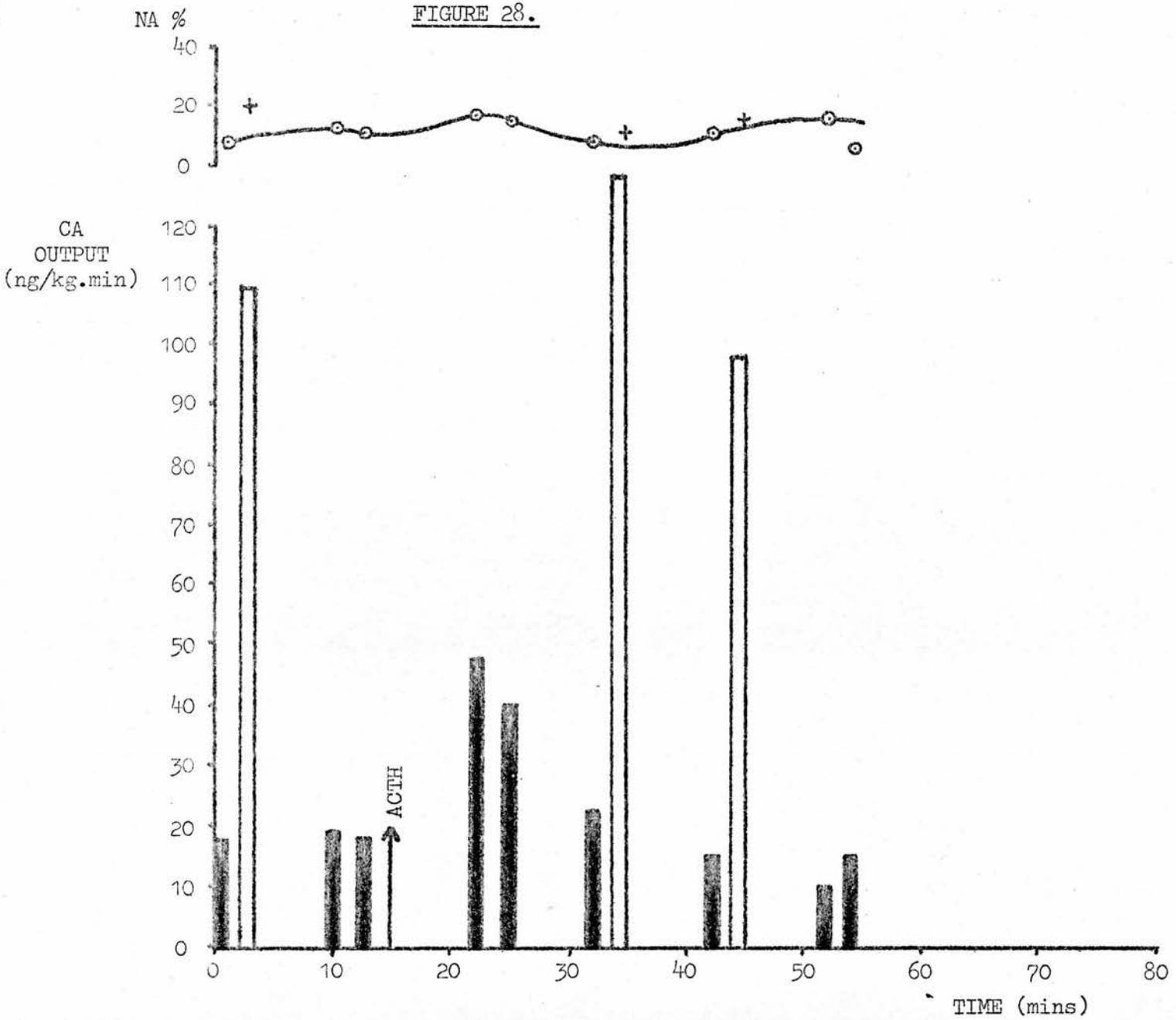


FIGURE 29.

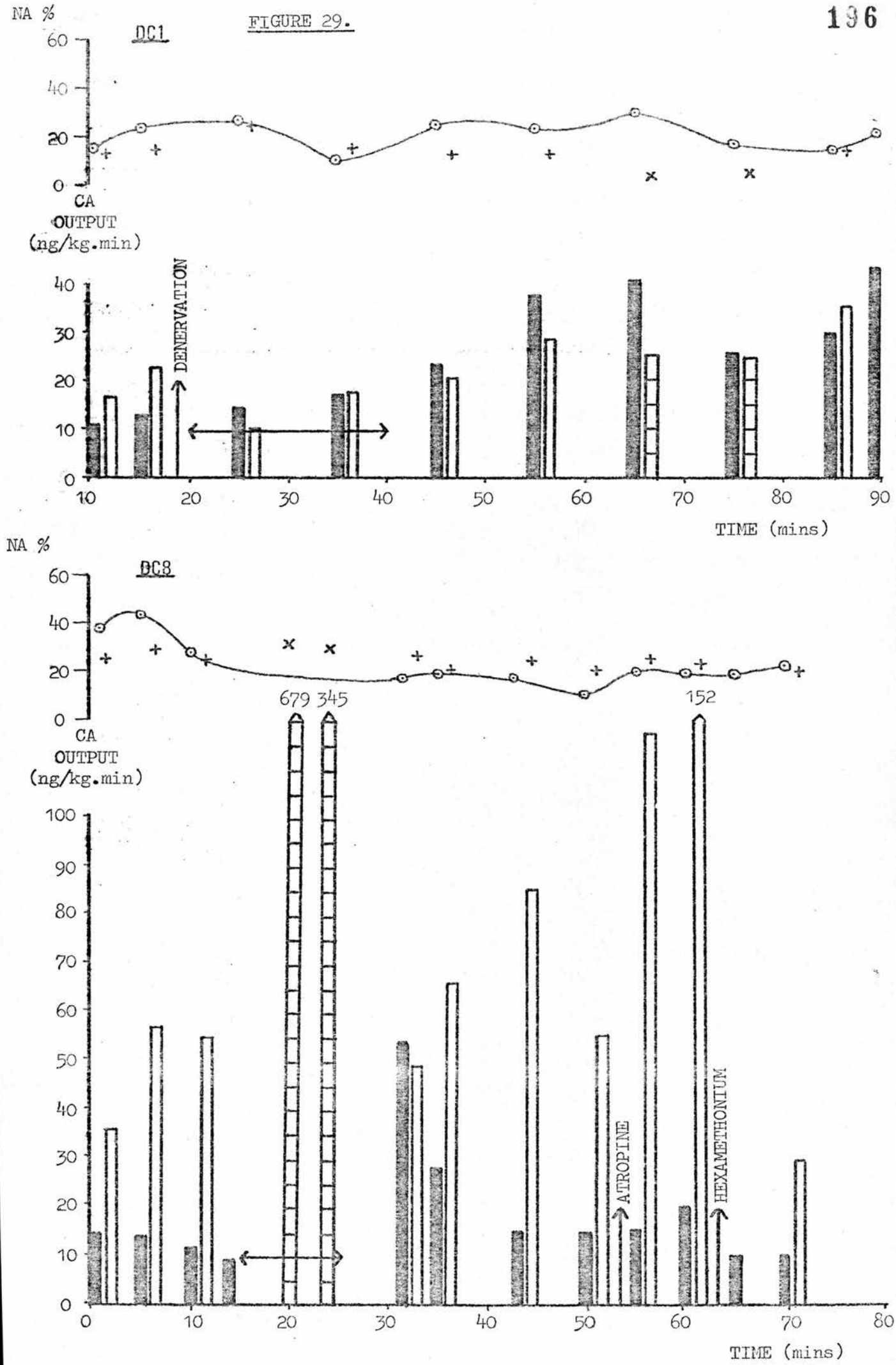
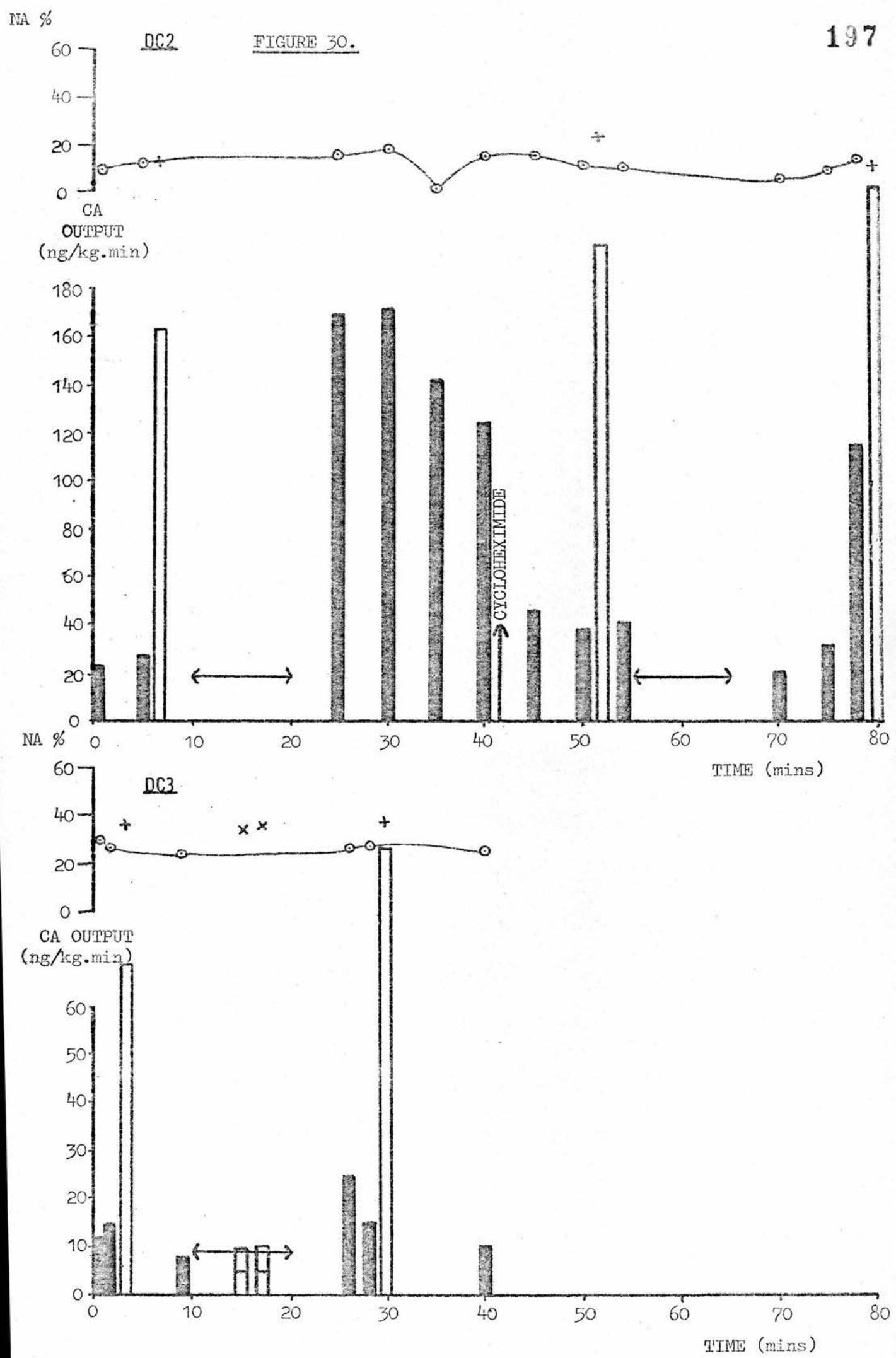

NA %
DC1
60
40
20
0
CA
OUTPUT
(ng/kg.min)
DENERVATION
40
30
20
10
0
10
20
30
40
50
60
70
80
90
TIME (mins)
NA %
DC8
60
40
20
0
679 345
152
CA
OUTPUT
(ng/kg.min)
100
90
80
70
60
50
40
30
20
10
0
ATROPINE
HEXAMETHONIUM
0
10
20
30
40
50
60
70
80
TIME (mins)

FIGURE 30.


NA %
DC2
60
40
20
0
CA
OUTPUT
(ng/kg.min)
180
160
140
120
100
80
60
40
20
0
CYCLOHEXIMIDE
NA %
0
10
20
30
40
50
60
70
80
TIME (mins)
DC3
60
40
20
0
CA OUTPUT
(ng/kg.min)
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
TIME (mins)

DC4 FIGURE 31.

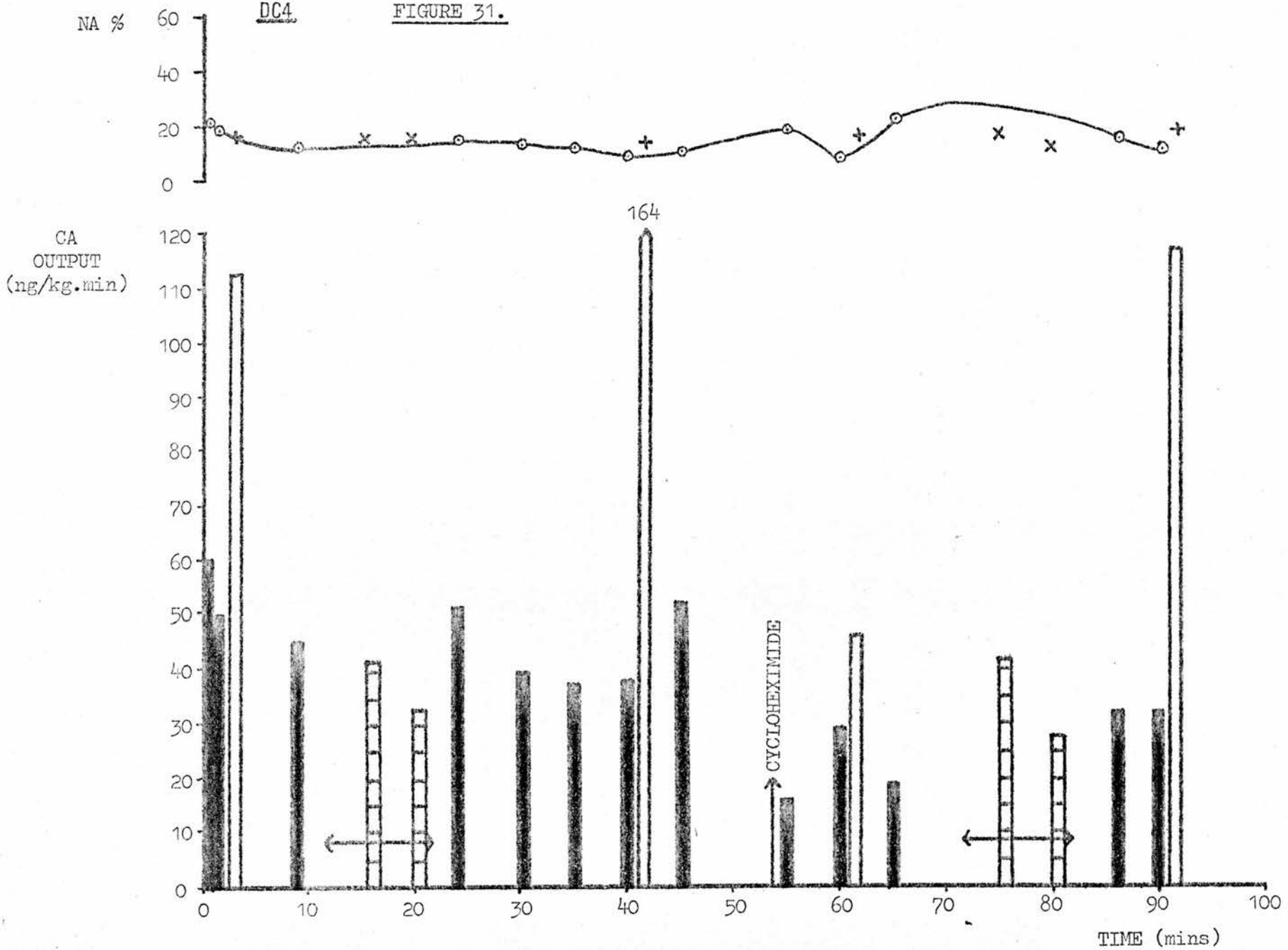


FIGURE 32.

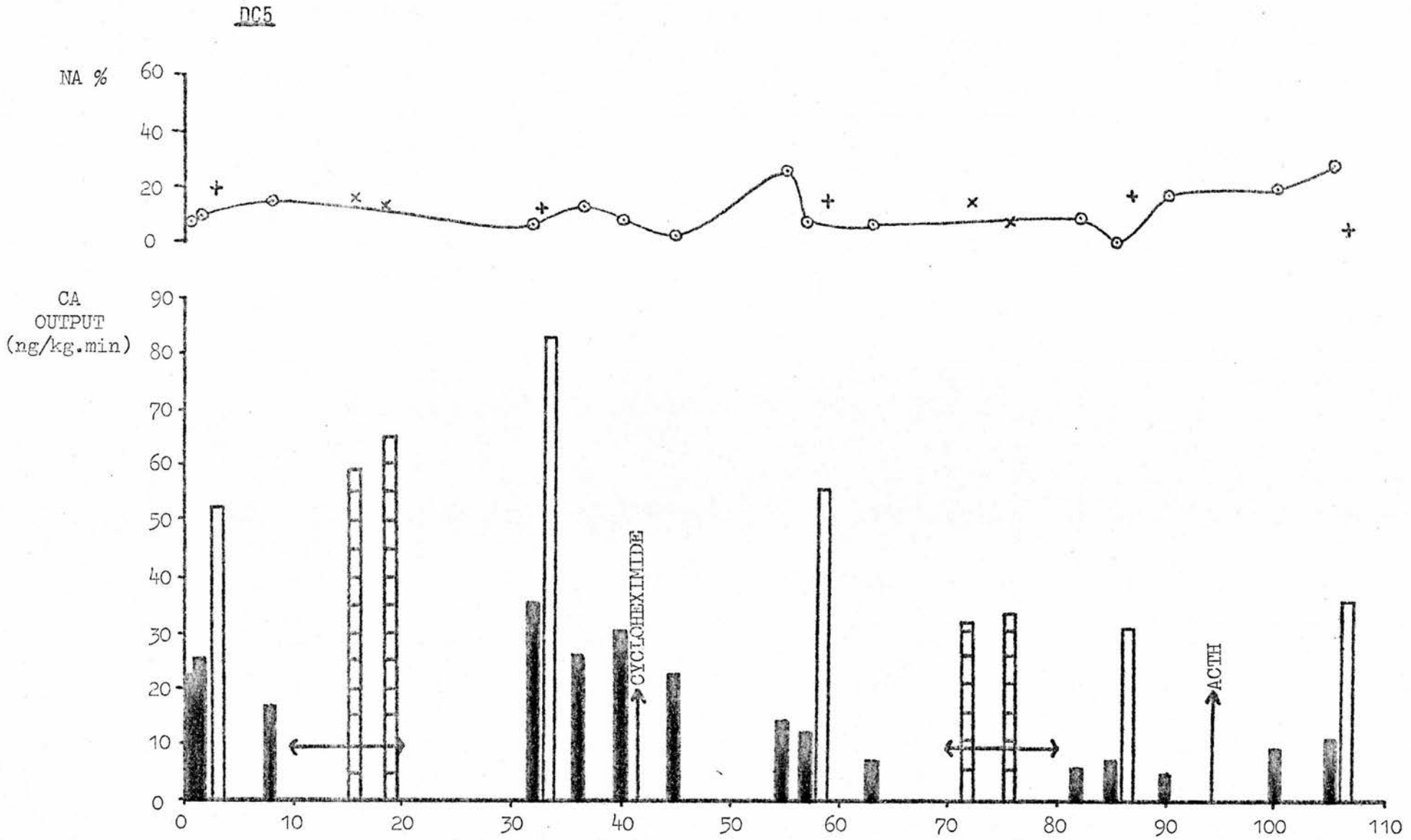

DC5
NA %
60
40
20
0
CA
OUTPUT
(ng/kg.min)
90
80
70
60
50
40
30
20
10
0
CYCLOHEXIMIDE
ACTH
0
10
20
30
40
50
60
70
80
90
100
110
TIME (mins)

FIGURE 33.

DC6

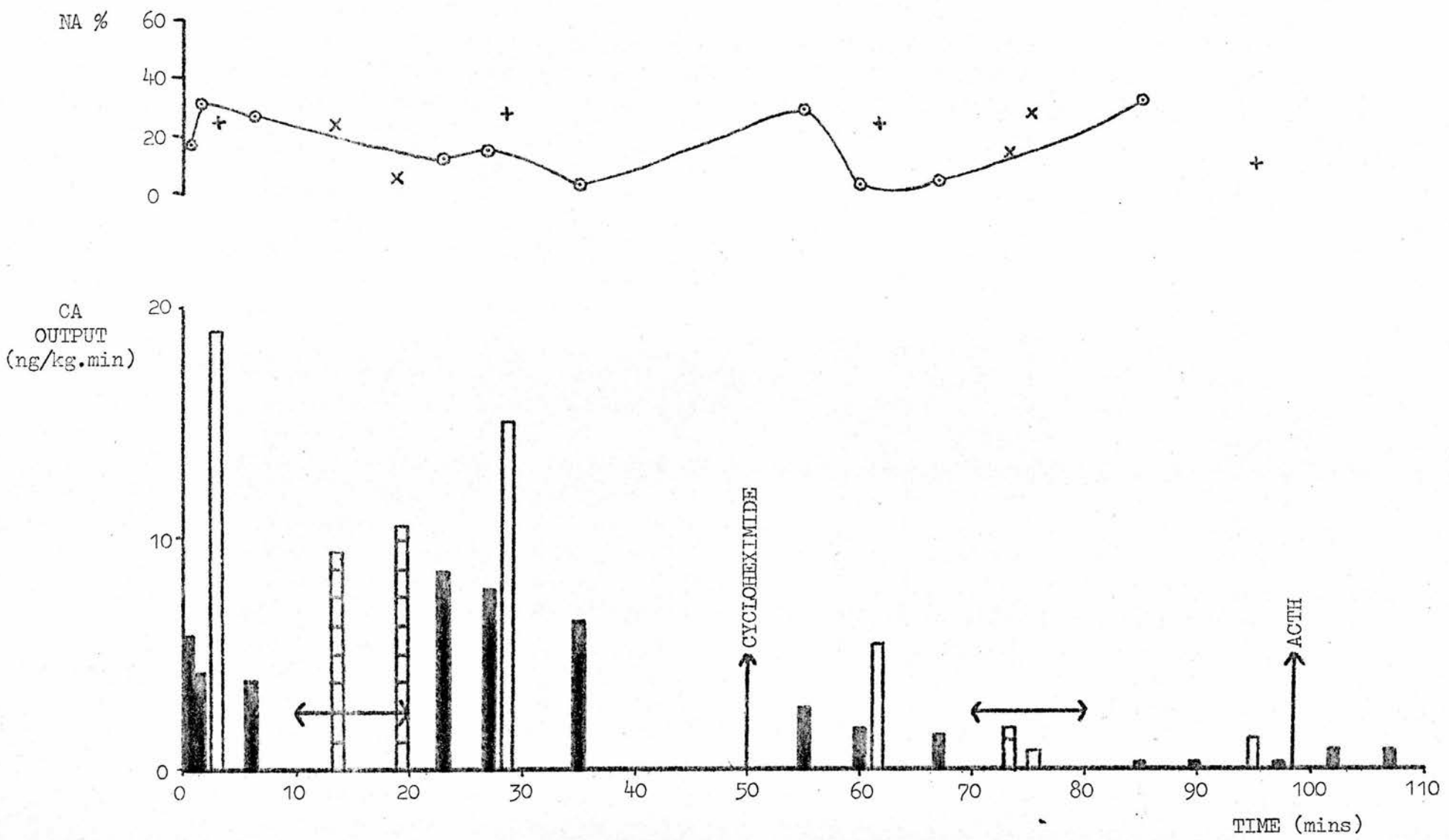


FIGURE 34.

DCZ

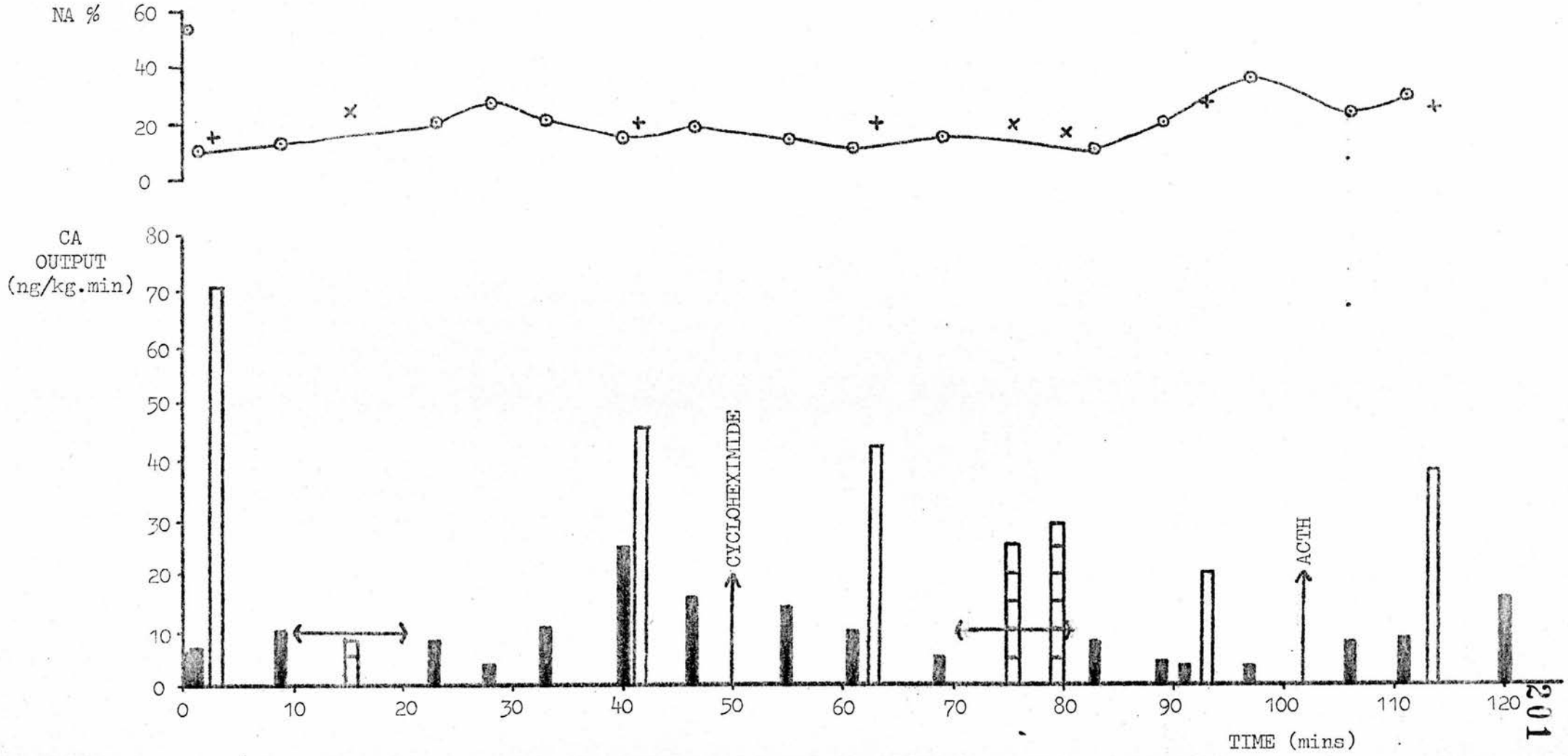
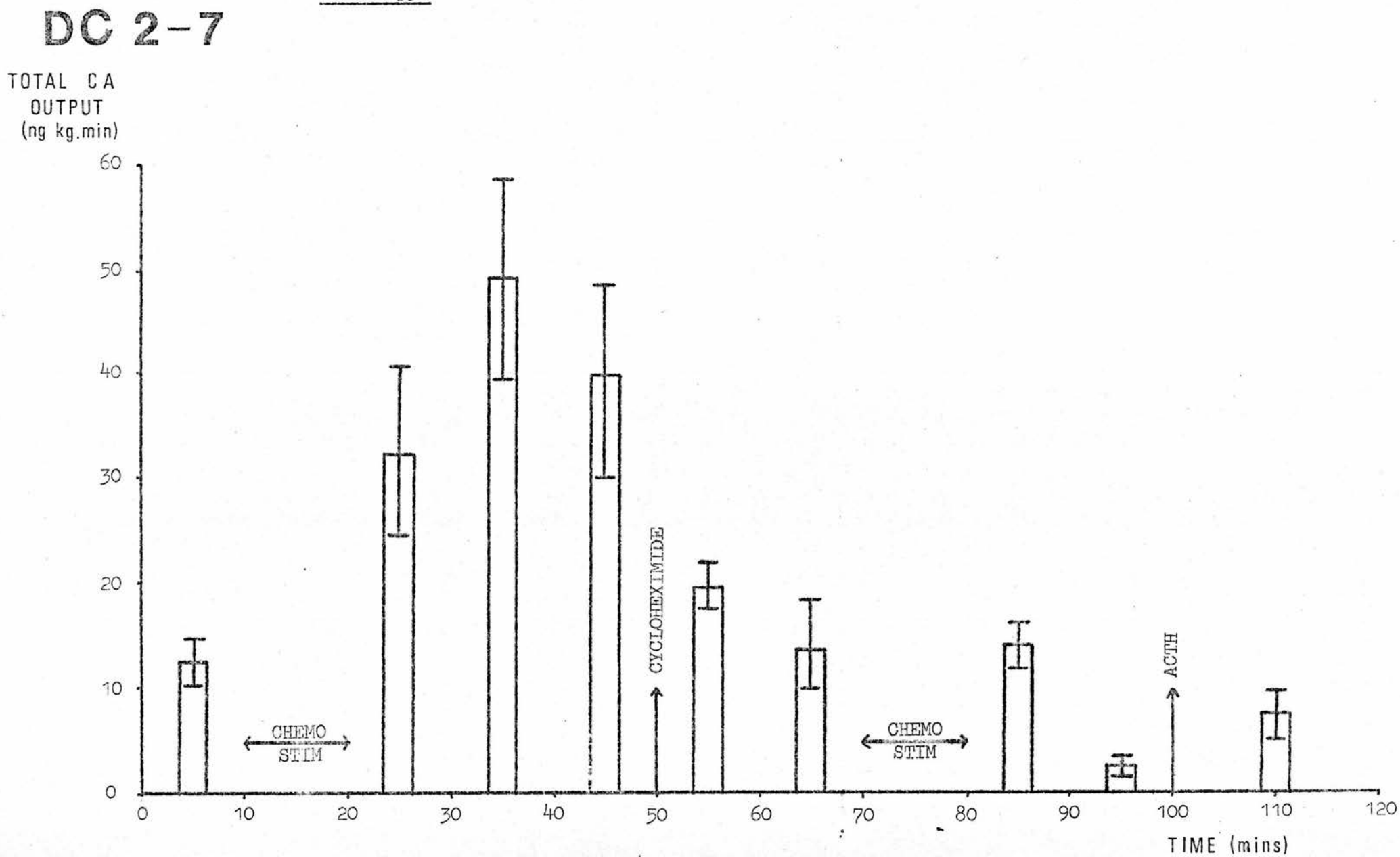


FIGURE 35.


DC 2-7
TOTAL CA OUTPUT (ng kg.min)
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
120
TIME (mins)
CHEMO STIM
CYCLOHEXIMIDE
CHEMO STIM
ACTH

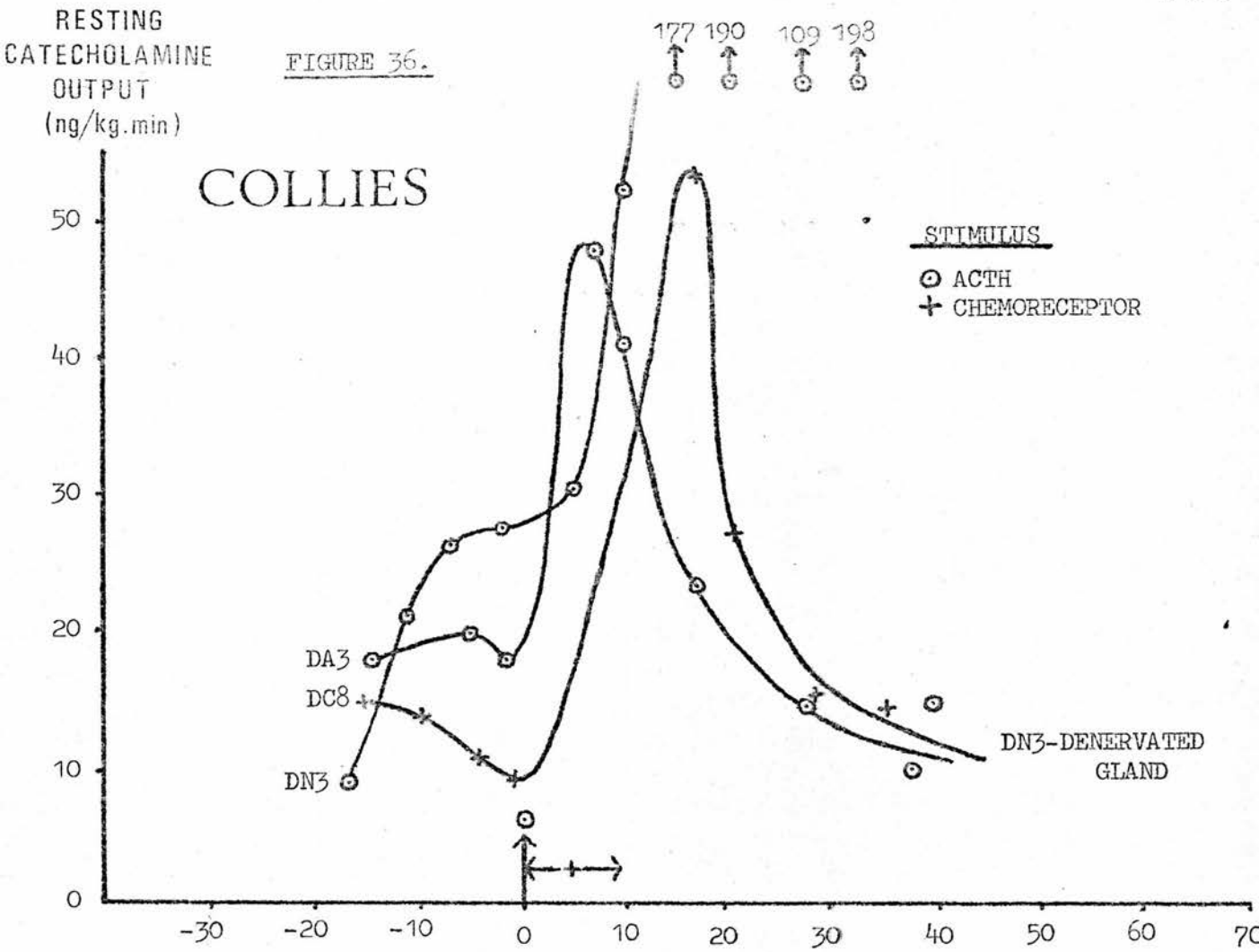
Difference between Greyhounds and Collie Sheepdogs

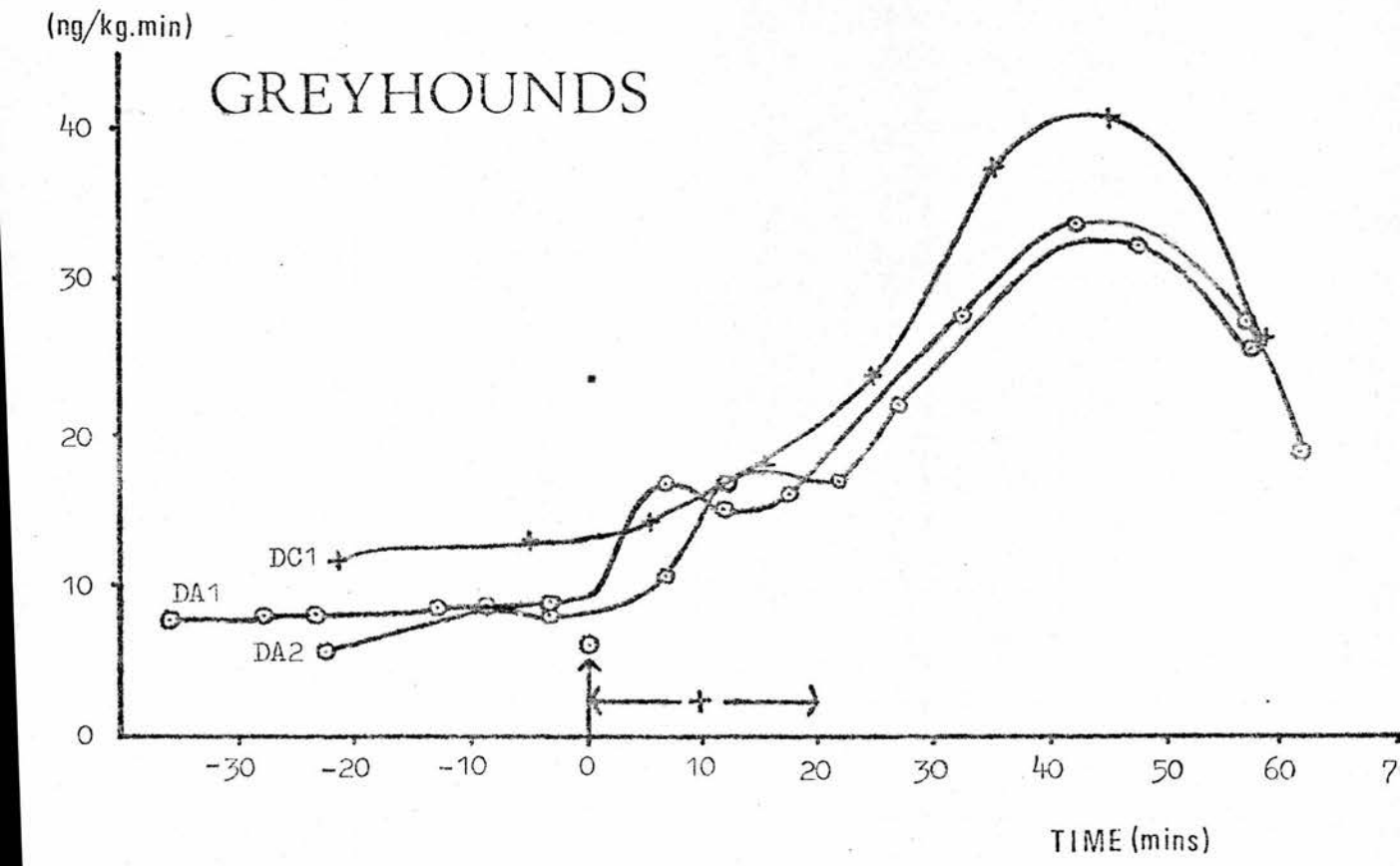
DA 1
DA 2 — Greyhounds
DC 1

DA 3
DN 3 — Crossbred Collie Sheepdogs
DC 2-8

We noticed that the humoral type response after Synacthen and prolonged chemoreceptor stimulation had a different timecourse in the two "breeds" of dog. The response of the crossbred collies was more rapid and of shorter duration than with the greyhounds. I have thus plotted DA 1, DA 2, and DC 1 separately from DA 3, DN 3 and DC 8 in Figure 36.

FIGURE 36.


RESTING CATECHOLAMINE OUTPUT (ng/kg.min)
177 190 109 198
COLLIES
STIMULUS
ACTH
CHEMORECEPTOR
DA3
DC8
DN3
DN3-DENERVATED GLAND
0
10
20
30
40
50
-30
-20
-10
0
10
20
30
40
50
60


(ng/kg.min)
GREYHOUNDS
DC1
DA1
DA2
0
10
20
30
40
-30
-20
-10
0
10
20
30
40
50
60
TIME (mins)

THRESHOLD OF THE HUMORALLY MEDIATED ADRENAL MEDULLARY RESPONSE TO CHEMORECEPTOR STIMULATION

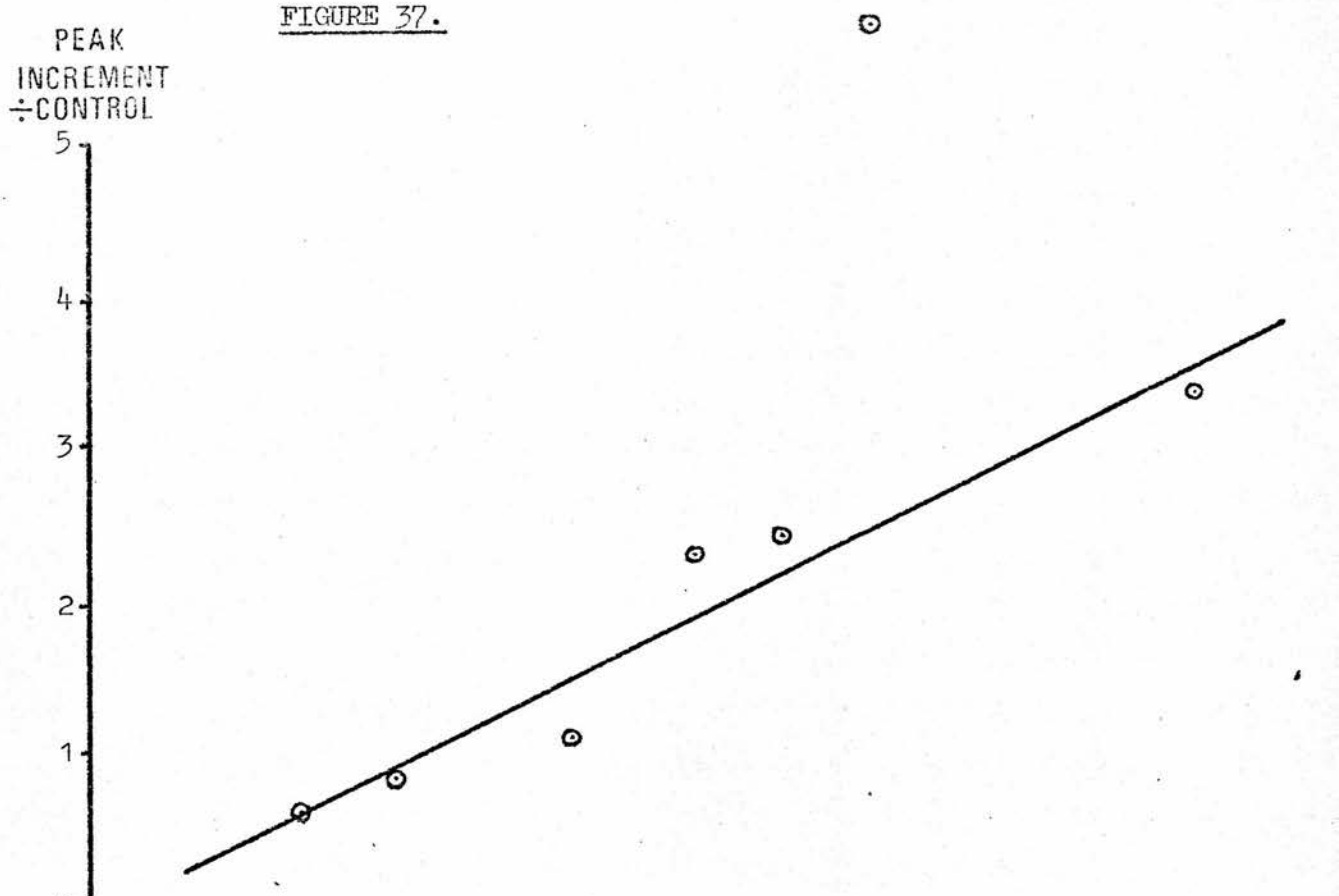
We wanted to see if there was a relationship between the degree of hypoxia achieved during the period of prolonged chemoreceptor stimulation and the magnitude of the humorally mediated component of the adrenal response.

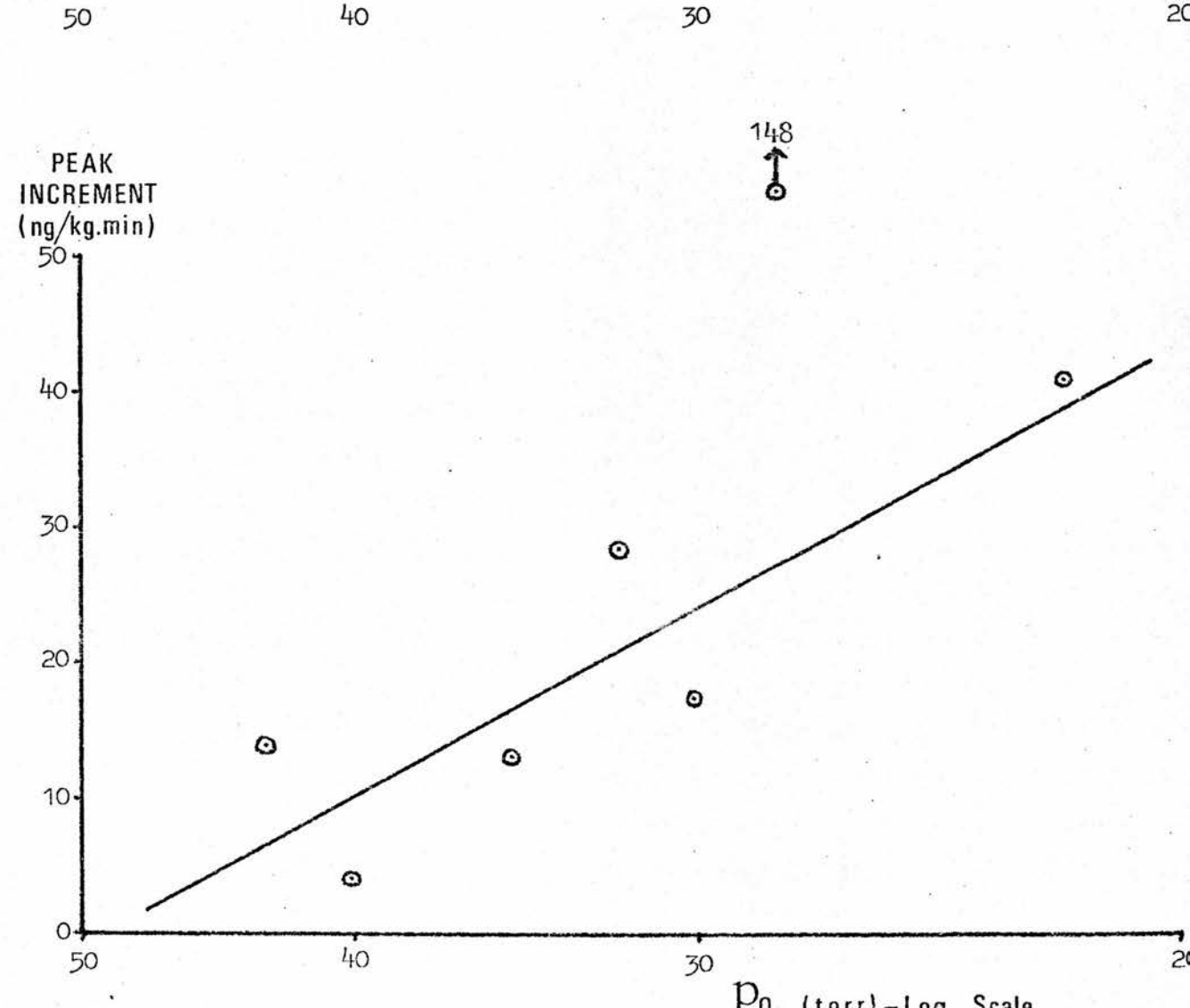
For each experiment (DC 1-8) I calculated the peak increment of the maintained adrenal response by subtracting the mean resting output before chemoreceptor stimulation (i.e. the controls) from the maximum resting output measured after the end of the prolonged chemoreceptor stimulus. (NB Only that half of the experiment before cycloheximide administration was considered).

The increments have been plotted against the logarithm of the minimum PO_2 reached during the chemoreceptor stimulus. I also plotted the ratio of the peak increment to the mean control resting output against the logarithm of the minimum PO_2. (Upper graph in Figure 37).

I used the logarithm of the minimum PO_2 as it gave a more linear relationship.

FIGURE 37.


PEAK
INCREMENT
÷CONTROL
5
4
3
2
1
0
50
40
30
20


148
PEAK
INCREMENT
(ng/kg.min)
50
40
30
20
10
0
50
40
30
20
P_{O_2} (torr) – Log_{10} Scale

ACTION OF CORTICOSTEROIDS ON ISOLATED PERFUSED CANINE ADRENAL GLANDS

The responses to the eight minute periods of corticosteroid infusion are recorded in the tables of results as the control output before infusion of the drug and:-

(a) The peak incremental release.

(b) The average incremental release over the four minute period of peak response.

These outputs are given as total catecholamines in ng /min

The percentage of noradrenaline in the control output and in the average increment (b) are also shown.

Hydrocortisone p 348 - 350

The 11 glands which had been obtained from heparinised animals, under pentobarbitone anaesthesia, fall into two groups:-

(a) "Unstressed" Glands from freshly anaesthetised dogs killed by exsanguination.

(b) "Stressed" Glands from animals that had been subjected to an acute cardiovascular experiment lasting 4 - 8 hours.

These two groups are plotted in Figure 38. For each group at three different concentrations of hydrocortisone, I have plotted the mean $\pm$ S.E.M. of the average incremental release. p 209

(a) Unstressed Glands

Glands A2-7, A11. (7 glands)

Hydrocortisone concentration:-	Release (Total Catecholamine)	
30 ug /ml	44 ± 14 ng /min	(n = 9)
50 ug /ml	286 ± 53 ng /min	(n = 8)
100 ug /ml	635 ± 92 ng /min	(n = 10)

(b) Stressed Glands

Glands A8-10, A12. (4 glands)

Hydrocortisone concentration:-	Release (Total Catecholamine)	
50 ug /ml	64 ± 46 ng /min	(n = 4)
100 ug /ml	186 ± 39 ng /min	(n = 5)
150 ug /ml	301 ± 49 ng /min	(n = 3)

Aldosterone p351

In three glands (A9, A10, and A11), which were responsive to the above order of concentration of hydrocortisone, no response was seen with aldosterone in concentrations of 5 - 20 ug /ml.

Deoxycorticosterone p352

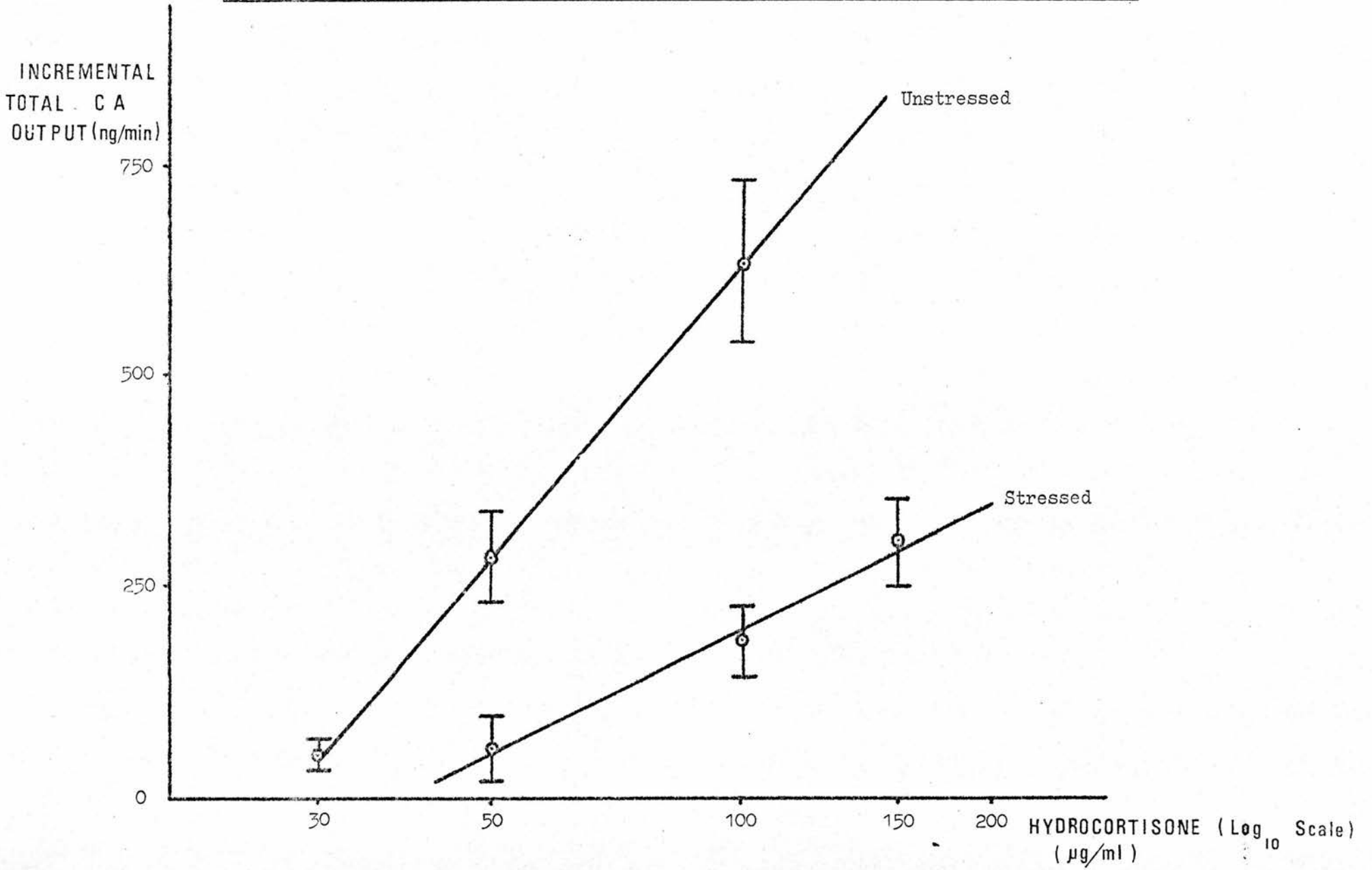
D.O.C. was shown to release adrenal catecholamines (n = 6) in 3 glands, in concentrations of 40 - 100 ug /ml.

Cycloheximide

We subjected three glands to ten minutes perfusion with Locke's solution containing cycloheximide (1 mg/ml.) and observed no change in the output of catecholamines.

FIGURE 38.

Response of Isolated Perfused Canine Adrenal glands to infusions of Hydrocortisone.



OBSERVATIONS ON ADRENAL BLOOD FLOW IN OUR WHOLE ANIMAL EXPERIMENTS

Adrenal Blood Flow After Synacthen Administration

Other workers had reported that adrenal blood flow increases following Synacthen administration (e.g. Edwards, Hardy and Malinowska, 1975). I have plotted the adrenal flows in experiments DA 1-3 and DN 3 (Figure 39) and in each case there is a tendency for the flow to rise after Synacthen was given.

These graphs also show that the adrenal blood flows were maintained reasonably well throughout the main part of these experiments. This was in spite of a gradual fall in blood pressure and is probably in part, due to the decreasing viscosity (and haematocrit) of the blood as the losses were replaced by Dextran.

Adrenal Blood Flow Response to Splanchnic Nerve Stimulation

For DN 1-3 I have shown the ratio of the adrenal blood flow during nerve stimulation to that flow during the control collection (Figure 40). Most points lie on the unity line and, thus, there appears to be no appreciable change in gland resistance during splanchnic nerve stimulation.

Reflex Vasoconstriction in Adrenal Gland During Baroreceptor Tests

We had performed a large number of baroreceptor tests in the dog and I thought that it would be interesting to see if there was any change in adrenal gland vascular resistance during these tests. The blood vessels might have responded passively to the reflex increase in systemic blood pressure and thus dilated with a consequent decrease in resistance. On the other hand, the sympathetic innervation to the adrenal blood vessels could have increased adrenal vascular resistance

FIGURE 39.

SYSTEMIC BLOOD PRESSURE AND ADRENAL BLOOD FLOW DURING CONTROL COLLECTIONS IN 4 DOGS
(given ACTH 1 IU/kg)

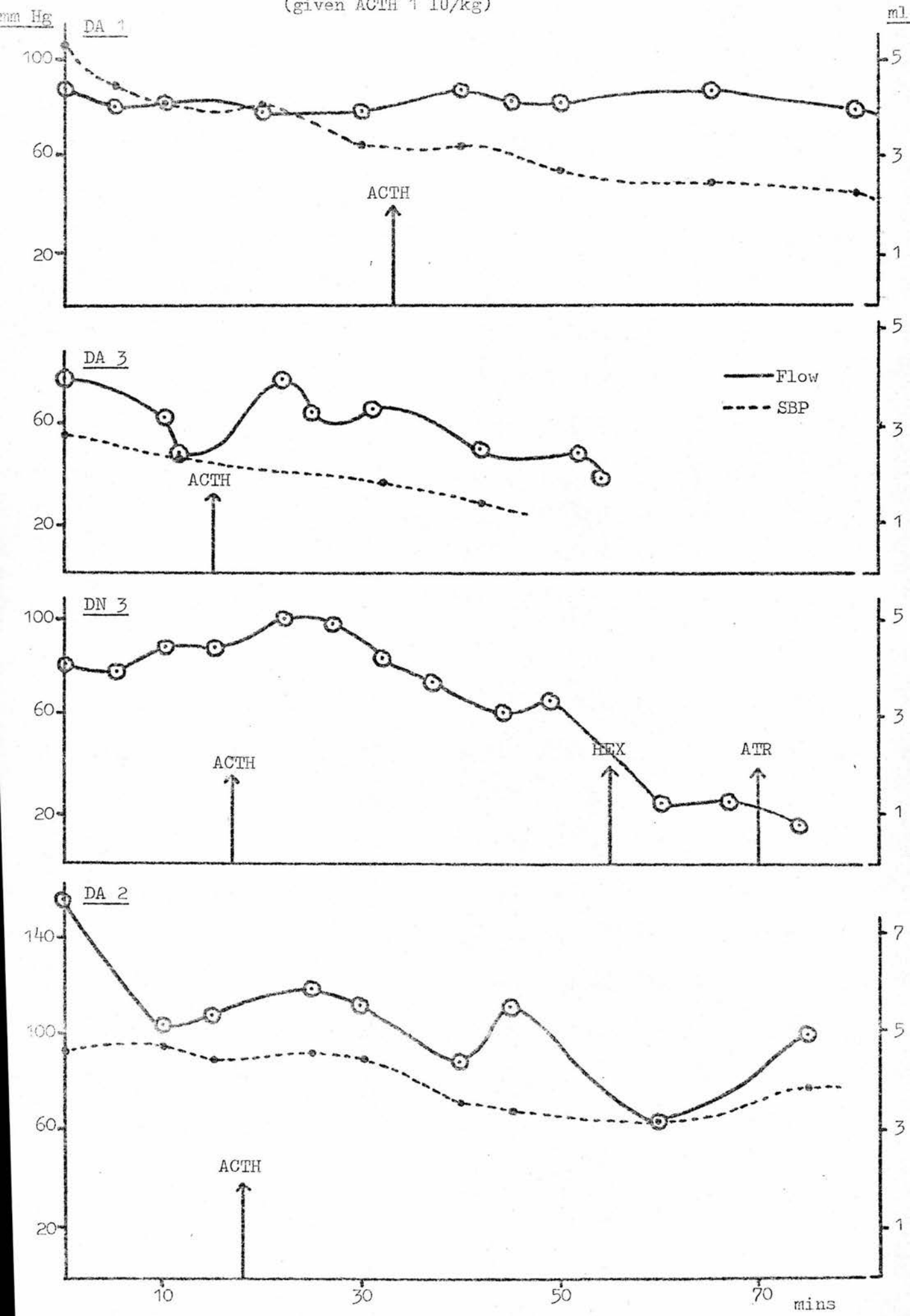
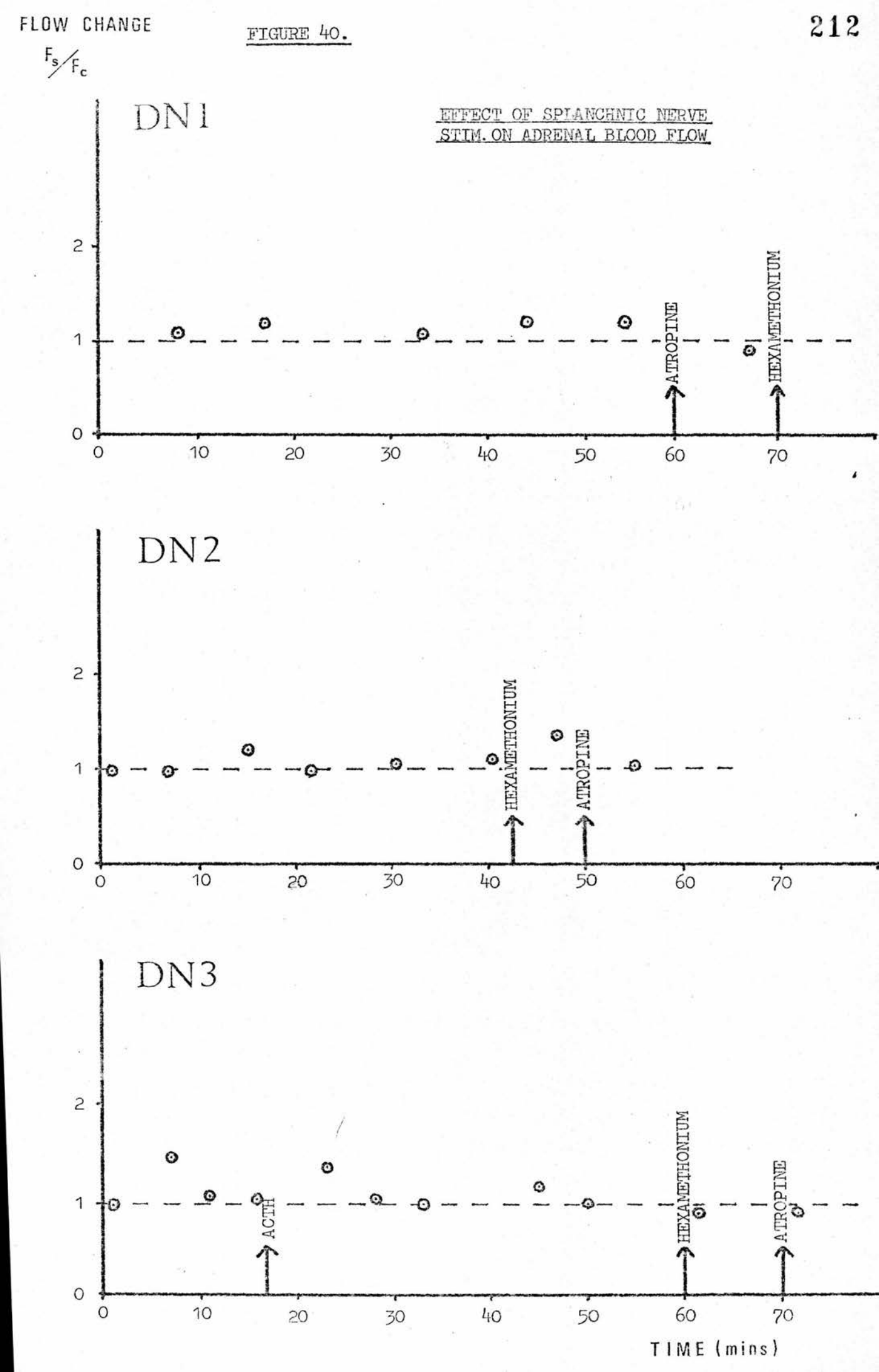


FIGURE 40.


FLOW CHANGE
F_s/F_c
DN1
EFFECT OF SPLANCHNIC NERVE STIM. ON ADRENAL BLOOD FLOW
ATROPINE
HEXAMETHONIUM
DN2
HEXAMETHONIUM
ATROPINE
DN3
ACTH
HEXAMETHONIUM
ATROPINE
0
1
2
0
10
20
30
40
50
60
70
TIME (mins)

as part of the vasoconstrictor response to a fall in carotid sinus pressure.

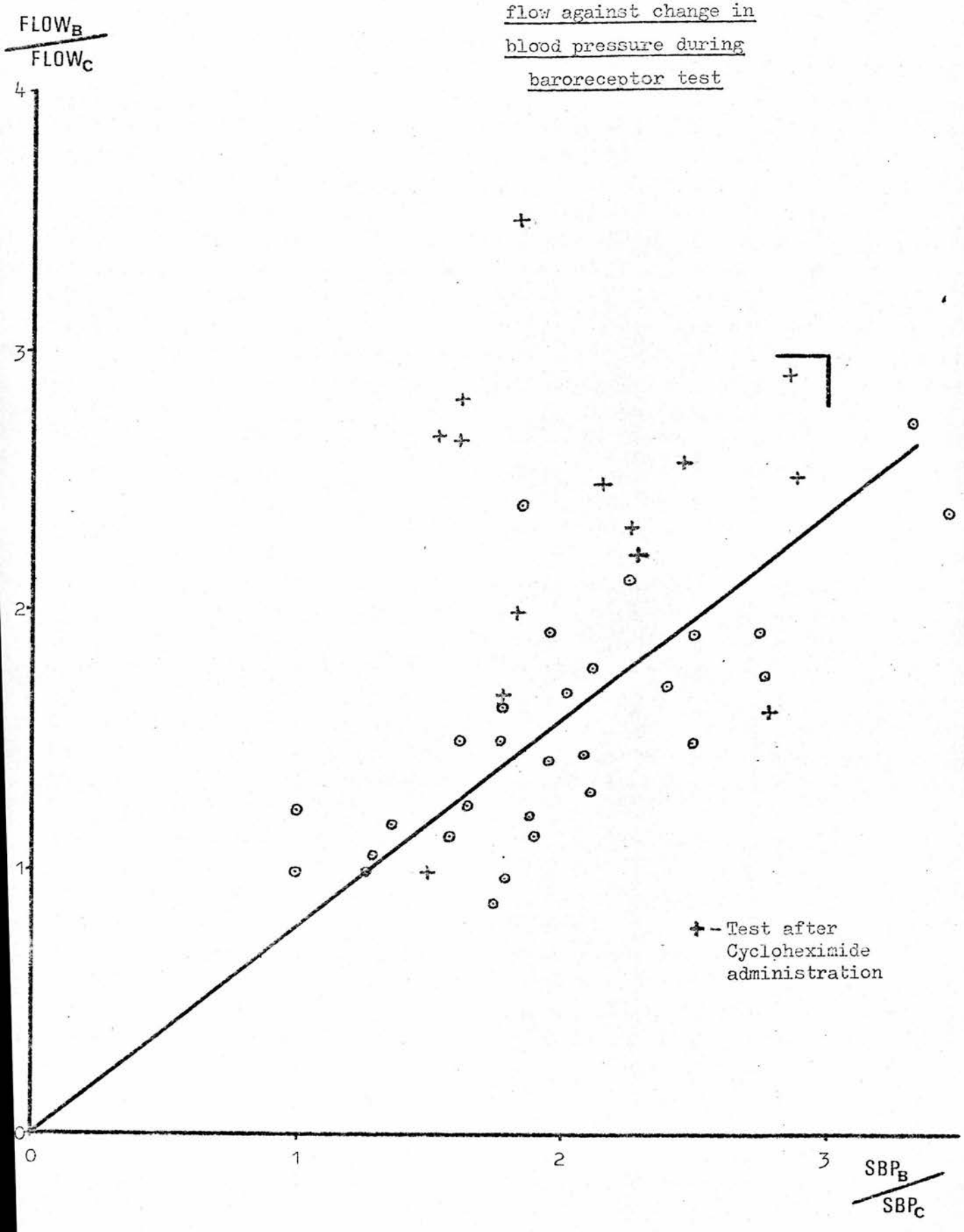
Our available data was the systemic blood pressure and the adrenal blood flow before and during the tests. Since the resistance of the dog adrenal glands and collecting tubing would vary with each experiment, I have plotted for each test the ratio of the systemic blood pressure during, to that before the test against the ratio of the adrenal flow during to that before the test (Figure 41). Pressure is directly proportional to flow at constant resistance and thus, if there is no change in resistance during the test, the points should lie around the line at 45° to both axis (i.e. the line with a gradient of one).

However, most points lie below this line (ignore tests performed after cycloheximide) and thus there must have been an increase in adrenal gland resistance during the baroreceptor tests. This is most likely to be due to sympathetic vasoconstriction.

Cycloheximide appears to abolish any reflex vasoconstriction in the gland as the points tend to lie on or above the bissecting line. The latter would be expected if there was passive dilatation in response to the rise in blood pressure. It does not seem to be a generalised effect on vasoconstriction as the systemic blood pressure responses to baroreceptor tests are not abolished by cycloheximide.

FIGURE 41.

Change in adrenal blood flow against change in blood pressure during baroreceptor test



ADRENAL BLOOD FLOW, CORTICOSTEROID CONCENTRATION AND CATECHOLAMINE OUTPUT

Our isolated gland experiments had demonstrated that the secretion of catecholamines from the adrenal medulla was influenced by the concentration of corticosteroids perfusing the gland. In the intact animal this concentration is determined not only by the release of corticosteroids from the adrenal cortex but also by the blood flow through the gland. Thus the transient increase in flow during a baroreceptor test would be expected to reduce any humorally mediated component of catecholamine secretion. Normally this phenomenom would tend to be obscured by the neuronally mediated catecholamine release in response to the reflex stimulus. However, in experiment DC 1 the adrenal gland was denervated and during the period of the delayed release the catecholamine output can be seen to fall in response to the reflex tests. (See Figure 29). p196

I have also looked for evidence of the effects of adrenal gland flow on catecholamine output in our other two experiments in which serial baroreceptor tests were superimposed upon a humorally mediated adrenal response. (i.e. DA 1 and DA 2)

By making two assumptions, I predicted the expected size of the output during the baroreceptor tests which were performed after corticotrophin administration. One was that our experimental conditions were sufficiently stable and our baroreceptor tests were sufficiently reproducible, for us to assume that any change in resting or test output was due to the corticotrophin administration. (See controls for DA 1 and DA 2). p193-4 The other was that the increased concentration of corticosteroid would potentiate by the same factor, both the resting output and the reflex release.

I have plotted for DA 1 and DA 2 the ratio of each test output after Synacthen to the mean of the test outputs before Synacthen against the ratio of each control collection to the mean of the pre-Synacthen controls. (i.e. I have plotted B ÷ Bm against C ÷ Cm see Figure 42).

For each test, using the C ÷ Cm axis, I have also plotted the increase in flow as the ratio of the test flow to the control flow. (F ÷ Fm)

In DA 1, there is a negligible change in flow during the baroreceptor tests and the points are seen to lie on the 45° line which is to be as expected if the resting and test catecholamine outputs are potentiated equally.

However, in DA 2, in which the flow nearly doubles during the tests, the test catecholamine outputs are seen to be less than predicted.

In a situation of stress involving hypotension, not only will there be an increased coricosteroid release but also the fall in gland blood flow will further increase the concentration of steroids in the blood perfusing the adrenal medulla.

Walker, Zileli, Reutter, Shoemaker, Friend and Moore (1959) had published three experiments in which they subjected dogs to haemorrhagic shock by initially removing over 500 mls. of blood. I have shown their results in Figure 43 as total catecholamine output and adrenal venous plasma 11 - hydroxycorticosteroid concentration. (I assumed a haemocrit of 50% to convert from their whole blood concentrations) In each experiment the blood pressure was maintained constant at 80 mm Hg after bleeding and it is interesting to note how the fluctuations in

GREYHOUNDS

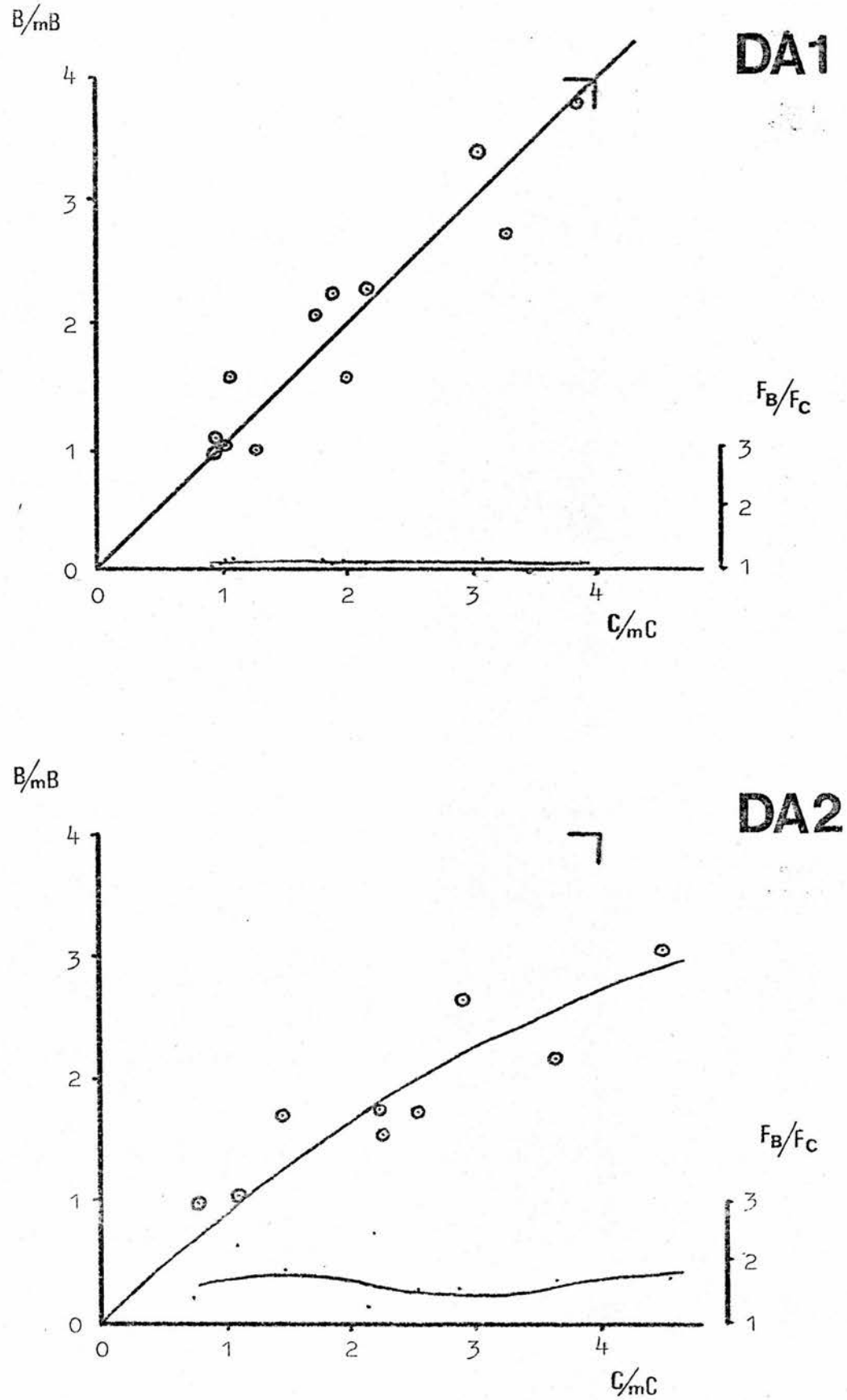


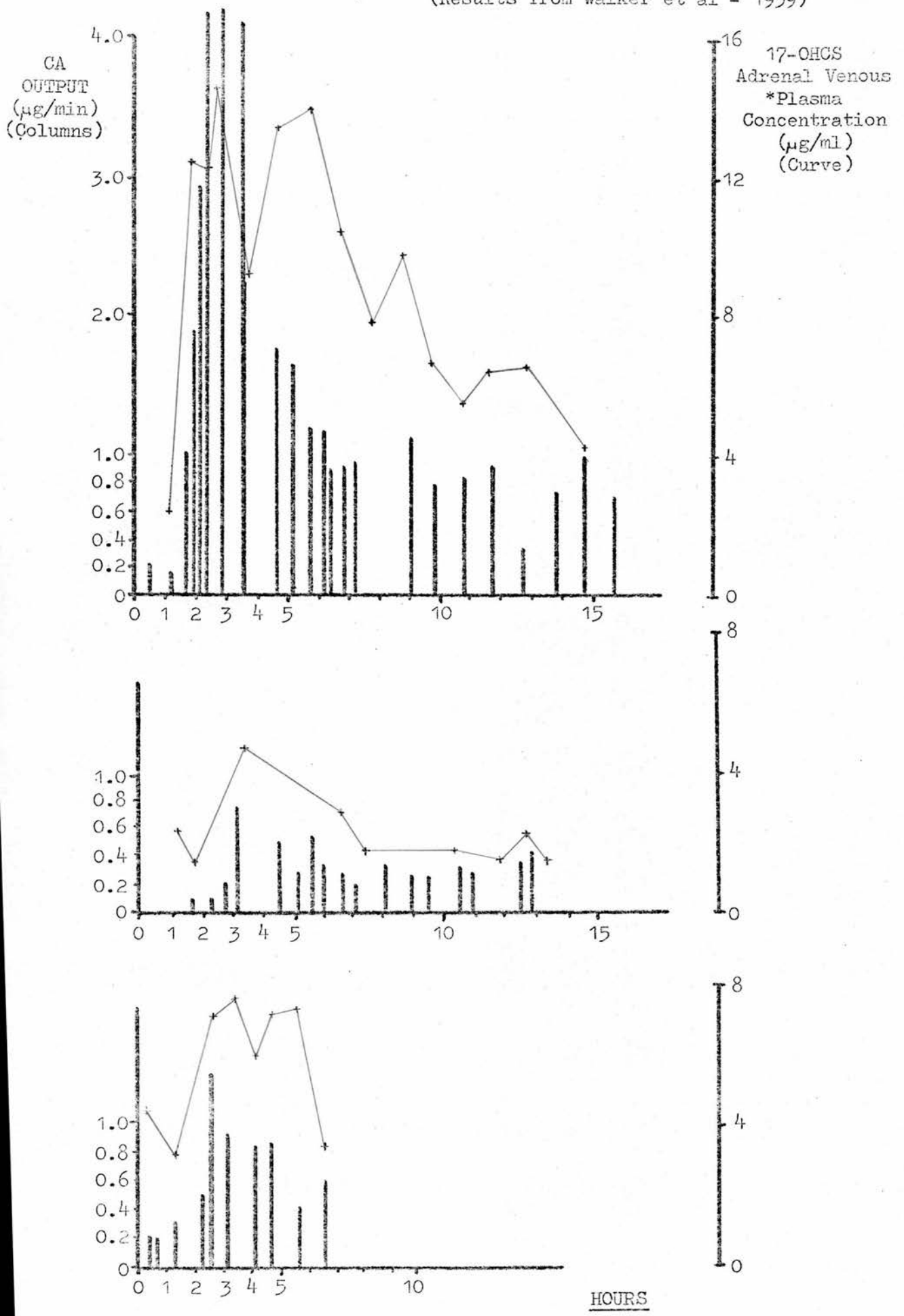
FIGURE 42.

catecholamine output parallel the changes in corticosteroid secretion. This is only circumstantial evidence but, as the stressful stimulus apparently remained constant there is no obvious reason why catecholamine release should follow the humorally controlled adrenocortical response - if the former is solely neuronally mediated.

It was very unfortunate that it was impractical for us to measure corticosteroid as well as catecholamine output at the time we were doing experiments DA 1-3, DN 3 and DC 1-8.

FIGURE 43.

(Results from Walker et al - 1959)



(* Calculated using Haematocrit of 50%)

EFFECT OF ADRENOCORTICAL ACTIVATION ON THE PERCENTAGE OF NORADRENALINE IN ADRENAL CATECHOLAMINE OUTPUT -TAKEN FROM DA1 - 3 and DC1 - 8

Resting Output	NA % (mean ± S.E.M.)	n
Control	20.01 ± 1.14	(33)
After Corticotrophin	17.44 ± 1.51	(19)
After Prolonged Chemoreceptor Stimulation	17.16 ± 1.22	(36)
Baroreceptor Incremental Release		
Control	21.63 ± 1.37	(19)
After Corticotrophin	18.73 ± 2.07	(16)
After Prolonged Chemoreceptor Stimulation	20.34 ± 1.93	(16)

DISCUSSION

SELECTIVE RELEASE EXPERIMENTS

Summary of Results

Within the limitations of our experimental conditions (i.e. anaesthetised animals and isolated perfused adrenal glands) our results demonstrate that:-

I. In Both Species

1. The carotid baroreceptor and chemoreceptor reflexes can individually evoke the secretion of adrenal catecholamines.

2. Both nicotinic and muscarinic stimulant drugs can release catecholamines from isolated adrenal glands and the responses to these drugs can be blocked by hexamethonium and atropine respectively.

II. In Dogs

No evidence for selective release was found.

1. The incremental releases mediated by the baroreceptor and chemoreceptor reflexes and by splanchnic nerve stimulation contained the same proportion of noradrenaline to adrenaline (i.e. 1:4) Furthermore it was equal to that found in the resting (i.e. control) output.

2. This proportion of noradrenaline to adrenaline was also found in the increments released in response to nicotinic and muscarinic stimulation.

III. In Cats

In this species we found evidence of selective catecholamine release with both reflex and pharmacological stimulation.

1. Baroreceptor stimulation released an increment containing predominantly noradrenaline (75% noradrenaline).

2. Chemoreceptor stimulation released an increment containing predominantly adrenaline (16% noradrenaline).

3. Both of the mean percentages were significantly different ($P < 0.01$)* from one another and from that in the resting output which contained 46% noradrenaline.

4. Nicotinic (hppTmA) stimulation released an increment which contained significantly ($P < 0.01$)* more noradrenaline (57% noradrenaline) than that released by muscarinic (methacholine) stimulation (34% noradrenaline). However, at none of the doses tested was there any evidence that either drug released solely one catecholamine.

These results suggest that with respect to the above reflexes and drugs, there is a species difference between the dog and the cat regarding the selective release of adrenal catecholamines (See Figures 44 and 45).

They are in agreement with the conclusions we reached after surveying the previous experimental work which has been published in our field. I have already discussed these studies in my Introduction and shall now only make some quantitative comparisons between them and our own observations.

* Student's "t" test.

FIGURE 44.

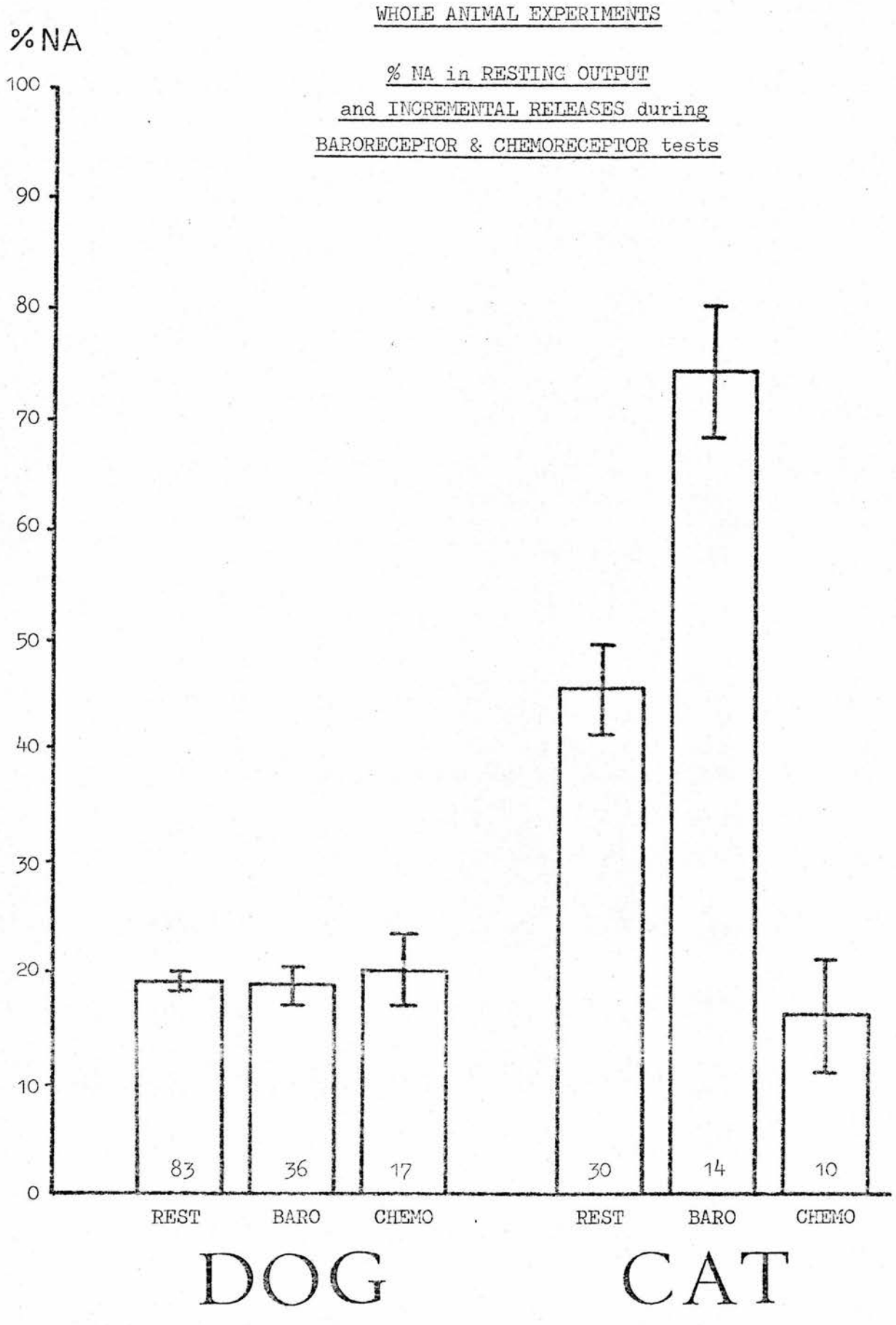
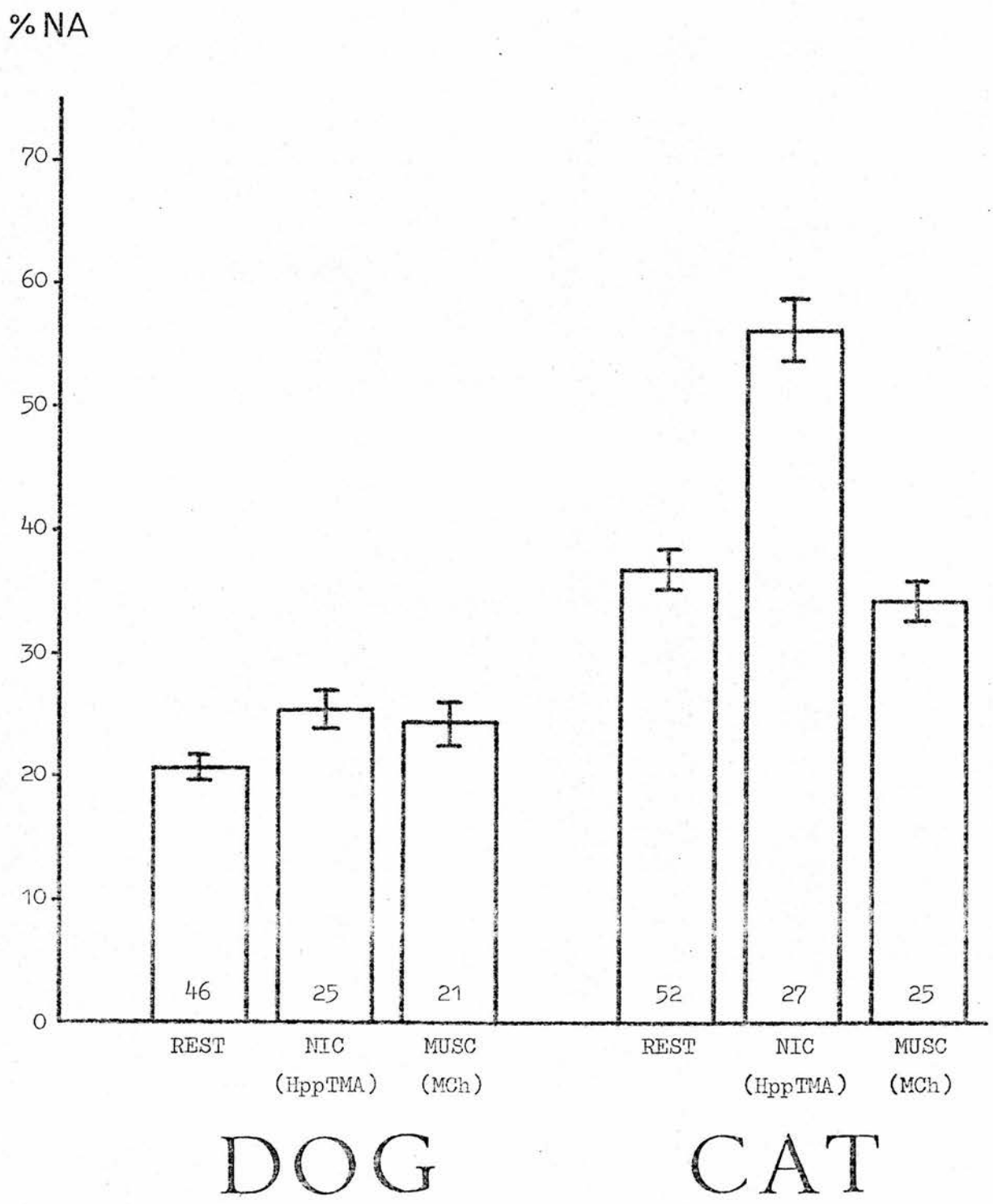

WHOLE ANIMAL EXPERIMENTS
% NA in RESTING OUTPUT
and INCREMENTAL RELEASES during
BARORECEPTOR & CHEMORECEPTOR tests
%NA
100
90
80
70
60
50
40
30
20
10
0
83
36
17
30
14
10
REST
BARO
CHEMO
REST
BARO
CHEMO
DOG
CAT

FIGURE 45.

ISOLATED ADRENAL GLAND EXPERIMENTS

% NA in RESTING OUTPUT
and INCREMENTAL RELEASES during
NICOTINIC and MUSCARINIC stimulation



THE DOG

Resting Secretion in our whole animal experiments (Dogs)

Much has been written on whether or not there is a resting "physiological" secretion from the adrenal medulla and about the effects of various experimental procedures and anaesthetics on this resting output (e.g. von Euler, 1956, De Schaepdryver 1959). Our resting output p168
was 14 ± 1 ng/kg. min, and I have listed the "resting" outputs found by some previous workers.

Workers	Anaesthesia	TOTAL CATECHOLAMINE ng/kg.min
Wada et al (1935)	Conscious	20
Lund (1951)	Chloralose-Barbiturate	9
Rapela & Houssay (1952)	Chloralose	13
Satake (1955)	Conscious	25
De Schaepdryver (1959)	Chloralose	6-26
Malmejac (1964)	Chloralose	46
Kayaalp + McIsaac (1968)	Pentobarbitone	4
Wurtman et al (1968)	Pentobarbitone	9

Reflex Release in our whole animal experiments (Dogs)

Our incremental releases in response to the carotid baroreceptor and chemoreceptor reflexes were 23 ± 5 and 15 ± 3 ng/kg.min respectively. p168
Not surprisingly these were much smaller than previous workers who used had found such stimuli as haemorhagic hypotension and asphyxia, (e.g. Rapela and Houssay 1952 - asphyxia gave an output of 437 ng/kg.min.) It has been our intention to use discrete reflex stimuli because we considered that these were more likely to reveal any capacity for selective release than the powerful stimuli in which the adrenal gland was "driven" to near maximum.

De Schaepdryver (1959) who used the more discrete stimulus of carotid occlusion obtained a similar sized incremental release of 14 ng/kg.min.

The incremental output in response to insulin hypoglycaemia found by Wurtman et al,(1968) was also of equivalent magnitude (39 ng/kg.min) to our discrete reflex stimuli.

We used electrical stimulation of the greater splanchnic nerve in order to examine the output from the adrenal gland under more intense stimulation than that mediated by the two carotid reflexes. With this stronger stimulus our mean incremental release of 168 ng/kg.min (range is up to 544 ng/kg.min) is comparable with that obtained by other workers e.g. Rapela and Houssay (1952b) - 308 ng/kg.min, De Schaepdryver (1959) - 350 ng/kg.min. Thus in the dog we were able to compare three different types of stimuli which gave mean incremental release from 11 to 168 ng/kg.min. However, even with this fifteen fold range of mean releases, there was no evidence of a significant deviation from the 1:4 noradrenaline to adrenaline ratio. p169

Pharmacological Stimulation in our Isolated gland experiments (Dogs)

The mean resting output from our perfused adrenal glands was 387 ng/min and this is well within the wide range of the control outputs quoted by Vogt (1965). Stimulation of the splanchnic nerve to her isolated Locke-perfused glands gave incremental releases ranging from 838 to 5300 ng/min which overlaps with our responses to hppTmA. In the spinal dogs of Kayaalp and McIsaac (1968), intra-aortic injections of microgram quantities of the following drugs gave mean releases of:- p171

Acetyl Choline	1.1 ug/min.	(21.3 ± 10.9% NA)
D M P P	1.5 ug/min.	(29.6 ± 13.6% NA)
Methacholine	2.2 ug/min.	(40.5 ± 13.3% NA)

(NB. The concentrations of the drugs perfusing their glands cannot be determined).

These figures compare with our mean incremental release of 757 ng/min. with hppTmA (2020 ng/min. when releases over 3000 ng/min. were included) and that with methacholine of 498 ng/min. I have commented in the Introduction that Kayaalp and McIsaac's mean value of 40% noradrenaline in the increment released by methacholine is not significantly different from the mean of 21% noradrenaline in the release with their nicotinic agonist. p172 p'65

IN CATS

Resting Secretion in our whole animal experiments (Cats)

Our control output was 6 ng/kg.min and contained 46% noradrenaline. p174

Fuerstein and Gutman (1971) who also used pentobarbitone anaesthetised cats and a very similar assay (see Introduction) reported a resting output of 5-6 ng/kg.min but it contained only 18% noradrenaline. The higher adrenaline percentage which they found may have been the result of hypoxia throughout their experiments as they do not mention putting their animals on artificial ventilation and/or oxygen after they had opened the abdomen. p60

Grant et al (1958) present some very interesting observations on the influence of anaesthesia on "resting" adrenal catecholamine output. Their cats were subjected to hypothalamic stimulation and they p59

admit that their animals were suffering from asphyxia, blood loss and other stresses. I have calculated the following figures from the data which they publish:-

Anaesthetic	Resting Output ng/kg.min		
	A	NA	%A
Chloralose-Urethane	60 ± 20	450 ± 80	11 ± 2
Urethane	70 ± 10	180 ± 30	33 ± 4
Dial	40 ± 10	2000 ± 400	2 ± 0.3

N.B. The three noradrenaline outputs are all significantly different ($P > 0.01$) from one another. (NB. means ±S.E.M.)

The overall resting output is very much higher than that found in Fuerstein and Gutman's and our own experiments. However, those earlier workers who used chloralose anaesthesia report "resting" outputs of over 190 ng/kg.min which contained between 71-81% noradrenaline. (Kaindl and von Euler 1951; Brücke, Kaindl and Mayer 1952; von Euler and Folkow 1953; Folkow and von Euler, 1954).

The finding by Grant et al that Dial anaesthesia was associated with a considerably greater noradrenaline output than was chloralose and/or urethane, could be explained by selective depression of the baro-receptor reflex by the latter two anaesthetics - as demonstrated for chloralose by Neil, Redwood and Schweitzer (1949). However, this is pure speculation and is not supported by the results of Duner (1953), who, in cats under pentobarbitone anaesthesia, obtained a resting output of 76 ng/kg.min containing 84% noradrenaline.

Reflex Release in our whole animal experiments (Cats)

Our incremental releases from selective reflex stimulation are of the same order of magnitude as those obtained with similar stimuli by other workers who used pentobarbitone anaesthesia (i.e. Fuerstein and Gutman, 1971 and Duner, 1954).

Fuerstein and Gutman found that during acute haemorrhage, the adrenal catecholamine output rose by 61 mg/kg.min while after a slower rate of bleeding it rose by 11 ng/kg.min and in both cases the increment contained at least 74% noradrenaline. (These figures compare with the increment of 5 ng/kg.min containing 75% noradrenaline which was obtained during our baroreceptor tests. p174

Dunér observed releases in the order of 30 ng/kg.min during hypoglycaemia in the cat but it took over forty minutes - (with a steadily falling blood glucose) to reach this output. However, Kaindl and von Euler employed chloralose anaesthesia and usually found that the incremental release from bilaterial carotid occlusion was between 50 and 200 ng/kg.min.

This order of magnitude difference in the responses to reflex stimulation may be due to the nature of the anaesthesia and for this reason we would have liked to perform some experiments in decerebrate animals.

Dunér and Kaindl and von Euler assayed their adrenal venous serum directly on the cat's blood pressure and fowl's rectal caecum. Kaindl and von Euler describe using peripheral blood samples to show that blood had no effect of its own on the test preparations.

However, their adrenal samples would have had high and probably variable concentrations of corticosteroids and I wonder if they took into account the potentiation by corticosteroids of the smooth muscle responses to catecholamines. (See Review by Ramey and Goldstein, 1957).

N.B. The permissive role of corticosteroids in the response of tissues to catecholamines is worth bearing in mind when interpreting some of the early work on adrenal discharge in which the responses to stimuli were compared before and after adrenalectomy.

Pharmacological Stimulation in our isolated gland experiments (Cats)

Douglas and Poisner (1965) observed that stimulation with nicotine yielded an increment of about 3500 ng/min which consisted of 55% noradrenaline. The increment obtained with pilocarpine was about 1500 ng/min and contained 4% noradrenaline/ while muscarine released a mixture containing 16% noradrenaline. These figures compare with our increment of 429 ng/min (57% noradrenaline) using hppTmA and that of 288 ng/min (34% noradrenaline) using methacholine. p 177

Douglas and Poisner make no mention of their standard errors and do not give the resting outputs from their glands. It is very probable that, like us, they found the basal secretion of the isolated perfused cat adrenal gland to be very low.

Hexamethonium resistance of the Cat chemoreceptor reflex P 181

Our results from Cats 4 and 5 indicate that both the blood pressure and adrenal medullary responses to chemoreceptor stimulation are resistant to doses of hexamethonium (2-6 mg/kg) which abolish both these components of the baroreceptor reflex.

Furthermore, Tenney (1956) found in cats that hexamethonium did not inhibit the adrenal catecholamine discharge nor the brisk hypertensive response to breathing 12-33% carbon dioxide.

We found these observations fascinating as they are compatible with the idea that the chemoreceptor reflex may be transmitted via predominantly muscarinic receptors while the baroreceptor reflex is transmitted via nicotinic receptors. The evidence for this hypothesis is as follows:-

1. Our work has shown in the cat that the chemoreceptor reflex releases predominantly adrenaline while the baroreceptor reflex releases predominantly noradrenaline from the adrenal medulla.

2. We have confirmed the work of Douglas's group which has shown that muscarinic stimulation releases predominantly adrenaline while nicotinic stimulation releases a greater proportion of noradrenaline from the adrenal medulla.

3. Professor James Black*- (various personal communications) has some evidence that in comparison with nicotinic agonists, the specific muscarinic ganglion receptor agonist, McNeil A - 343, (Rosykowski 1961) has relatively greater constrictor effect on veins than on arteries. For this work, he used the constant volume (i.e. equal inflow and outflow) capacitance technique on loops of cat small intestine. (see p.283)

4. Finally, there is evidence from the work of Pelletier, Shepherd and Webb - Peploe (see Pelletier 1972, Pelletier and Shepherd 1972 and Webb - Peploe and Shepherd 1968) that the carotid chemoreceptor reflex has a greater influence on hind limb veins than has the baroreceptor reflex. These experiments were performed on dogs and I will refer to this later.

One can see two distinct patterns connecting these four separate sets of observations which are:

(a) chemoreceptors - muscarinic receptors - adrenaline - veins

(b) baroreceptors - nicotinic receptors - noradrenaline - arteries

Furthermore, an important observation from our pharmacological studies on cat glands was that, although muscarinic and nicotinic stimulation released different proportions of the two catecholamines, there was no evidence, at any dose, that either of the agonists released only one catecholamine.

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Therefore, it seems unlikely that the difference in the distribution of the two types of receptor is simply related to whether the adrenal medullary cells are noradrenaline or adrenaline secreting. On the other hand, the proportions of the two catecholamines released with pharmacological stimuli are similar to those released in response to reflex stimulation and thus it is probable that the distribution of muscarinic and nicotinic receptors is related to the two different reflex pathways. The selective inhibition by hexamethonium of the predominantly noradrenaline releasing, and presumably nicotinic, baroreceptor reflex while leaving the predominantly adrenaline releasing chemoreceptor response undiminished, is further evidence for the existance of two different pathways.

However, atropine in doses up to 1 mg/kg did not block either the adrenal or the blood pressure response to chemoreceptor stimulation. Thus it seems unlikely that the pathway is only via muscarinic receptors. Perhaps in the cat, the chemoreceptor pathway has both types of receptor while the baroreceptor reflex is via nicotinic receptors alone.

Although we have found no evidence of selective catecholamine release in the Dog, there is evidence to suggest that in this species as well, there is some difference between the two carotid reflex pathways in the distribution of nicotinic and muscarinic receptors.

I have already mentioned the work of Shepherd's group on dogs p231 regarding the relatively greater influences of the baroreceptor and chemoreceptor reflexes on arterial resistance and venous capacitance respectively. This fits in with Black's resultsusing specific ganglion agonists in cats.

Furthermore, we have observed in dogs that the systemic blood pressure and hind limb vasoconstrictor responses (constant flow arterial perfusion technique) to carotid chemoreceptor stimulation, but not the baroreceptor reflex, is hexamethonium (2-6 mg/Kg) resistant in a manner similar to that observed in cats.

Ungar reports having blocked the hexamethonium resistant component in dogs with atropine but it required very large doses (50 mg/Kg) which were one hundred times that necessary to block the cardiac effects of the vagus. Thus, there appears to be a difference between the ganglionic and the more peripheral types of muscarinic receptors, with regard to the action of atropine which is possibly due to a difference in the affinity constants at the two types of receptor. This may explain our failure to block the chemoreceptor reflex in cats with atropine alone when using doses up to 1 mg/Kg.

HUMORALLY MEDIATED RELEASE EXPERIMENTS

Our evidence for the participation of a humoral component, via the anterior pituitary-adrenocortical axis, in the control of canine adrenal catecholamine output can be divided into six parts.

1). We found that the release of catecholamines provoked by chemoreceptor stimulation outlasts the stimulus and usually continues to rise after its cessation, (see DC1-8). p190

2). This delayed component of the adrenal catecholamine discharge in response to chemoreceptor stimulation was seen in a denervated gland. Denervation abolished the immediate catecholamine release in response to chemoreceptor stimulation and the baroreceptor reflex, (see DC1).

3). Chemoreceptor stimulation has been shown by others (Anichkov et al 1960, Marotta 1972) to release corticotrophin from the pituitary gland.

4). We found that intravenous Synacthen releases catecholamines from both intact (see DA1-3) and denervated adrenal glands (see DN3). p190

5). The delayed release of catecholamines in response to both Synacthen (see DC5-7) and chemoreceptor stimulation (see DC2-7) is inhibited by cycloheximide (see Figure 35, p202) which has been shown to block steroid secretion (Garren, Ney & Davis 1965). There was no evidence that cycloheximide had any effect on neuronally mediated adrenal medullary release and it did not appear to influence the immediate response to either chemoreceptor stimulation or the baroreceptor reflex (see graphs of the individual experiments). Furthermore, the output from isolated perfused adrenal glands (n=3) was unaffect by ten minutes of perfusion with the drug at a concentration (i.e. 1mg/ml.) well above its predicted maximum blood level.

6). Hydrocortisone in concentrations between 30 and 150 ug/ml. was shown to increase the catecholamine output from the isolated Locke perfused adrenal gland in a dose dependent manner, (see Figure 38). A study of the literature put this order of concentration in perspective. In dogs during stress or after corticotrophin administration, the adrenal venous plasma concentration of corticosteroids (mainly hydrocortisone) is in the range 10-20 ug/ml. However, not all the blood passing through the gland goes through the cortex-medullary portal system as the gland has direct through channels such as the arteriae medullae. Thus it is very probable that the steroid concentrations in the cortical blood bathing the meduary cells are higher than those in the adrenal vein and well within the range 30-150 µg/ml. P20

The amount of free hydrocortisone in the peripheral blood is very dependent on the concentration and degree of saturation of the hormone's main transport protein, transcortin. However, the latter's binding sites are completely saturated by concentrations of hydrocortisone above 400 ng/ml plasma and thus the binding protein will not significantly affect the concentration of free glucocorticoid in the blood perfusing the adrenal medulla.

It is possible that the artificial conditions of our isolated gland preparation altered its sensitivity to corticosteroids. However, the work of Vogt (1965) indicates that our perfusion technique would have been more likely to reduce rather than increase the sensitivity of the adrenal medullary cells.

Catecholamines have been shown to release corticotropin from the anterior pituitary (Vogt 1944 & Long 1952) and this is one explanation for the signs of Cushing's syndrome seen in some patients with phaeochromocytoma. (Another explanation is the production of corticotrophin-like peptides by the tumour). These symptoms disappear on removal of the tumour (Williams et al 1960). We therefore, considered the possibility that in our experiments corticotrophin was being released by catecholamines

secreted from the other adrenal gland during the chemorecepter stimulus. This mechanism was excluded by an experiment in which, with the other (i.e. right) adrenal gland denervated, prolonged chemoreceptor stimulation still gave the typical maintained output from the cannulated gland (DC 8).

We performed all these experiments on dogs and thus it is only in this species that we have evidence of a hormonal component participating in adrenal catecholamine control.

The association of phaeochromocytoma and Cushing's syndrome has led to some patients with phaeochromocytoma being subjected to tests of anterior pituitary-adrenocortical function and deaths due to severe hypertensive crisis have followed the administration of corticotrophin to such patients, (Moorhead, Caldwell, Kelly and Morales, 1966 ; Cowley, Montgomery and Wellbourn, 1970). These deaths can be explained by our own observations. Not only have we demonstrated the release of adrenal catecholamines in response to Synacthen but we have also shown that deoxycorticosterone (DOC), in the order of concentration that we expect in the adrenal gland after 11-B-hydroxylation inhibition by metyrapone (see Jenkins et. al. 1958) , had a similar action to hydrocortisone on isolated adrenal glands. P208 Furthermore, corticosteroids are known to potentiate the peripheral action of catecholamines, (Ramey and Goldstein,1957)

Bloom, Edwards, Hardy and Malinowska (1976) have recently presented some work in which they claim that the response of the adrenal medulla to chemoreceptor stimulation is trivial, as well as finding no evidence in favour of a delayed humoral type component. However, they were using five week old calves and a technique very different to that employed in our experiments. It entailed subjecting conscious animals, each wearing a helmet, to periods (up to ten minutes) of pure nitrogen breathing. This is not a pure chemoreceptor stimulus, and as the calves were not vagotomised

it is very probable that secondary reflexes, such as those from lung stretch receptors, were involved. Their conclusions regarding the threshold of their small adrenal medullary response to hypoxia are probably unjustified because of the non-steady state conditions existing at the commencement of nitrogen breathing. The arterial oxygen tension would have been falling rapidly at the time when they detected the adrenal catecholamine discharge.

Furthermore, in their calves, the adrenal medulla would not have been fully mature (see West 1955) and examination of the earlier work from their laboratory (Comline and Silver 1966) shows that around the time of birth, the adrenal medulla of the calf undergoes profound changes in its response to hypoxia and related stimuli. The degree of methylation is known to be related to maturity and appears to depend on the cortical supply of glucocorticoids (see p. 69)

Thus comparisons between our experiments in adult dogs and their work on asphyxiated conscious calves may not be all that useful. This is especially so when one considers that, due to the presence of an intact vagus, secondary reflexes may influence the primary chemoreceptor reflex to the adrenal medulla in a fashion similar to the modification of the vasoconstrictor response.

However, it is interesting to note that the time course of the adrenocortical response to ten minutes of anoxia in the calf is very similar to that of the change in catecholamine secretion which we observed in the dog in response to corticotrophin and chemoreceptor stimulation. They also comment that the extent of this increase in corticosteroid output was equivalent to that seen after supramaximal doses of ACTH in calves of the same age.

In an earlier paper (Edwards et. al. 1975), they describe how the administration of cycloheximide (10 mg/Kg intravenously) inhibited the

steroidogenesis but not the increase in adrenal blood flow in response to an infusion of Synacthen.

I have already discussed the evidence that the amount of the methylating enzyme, PNMT, in the adrenal medulla is dependent on the delivery of very high concentrations of glucocorticoids from the cortex (see p. 69) We suggest that as catecholamine release may induce the synthesis of a new enzyme protein, the levels of methylating enzyme in the medulla may be related to the supply of cortical glucocorticoids through the mechanism of catecholamine release. This is compatible with the observation that tyrosine hydroxylase levels also fall after hypophysectomy and with a half life less than that of the fall after denervation (Mueller, Thoenen and Axelrod 1970).

We wondered whether glucocorticoids could have a preferential effect on adrenal secretion and alter the one to four, noradrenaline to adrenaline ratio. Thus it is interesting that the mean percentages of noradrenaline in the resting outputs after corticotrophin (17.2 $\pm$ 1.2%) p 220 are significantly lower ($P < 0.05$) than those in the control resting output (20.0 $\pm$ 1.1%). However, in keeping with our results on selective release there was no significant difference between the mean noradrenaline percentages in the neuronal releases mediated by the baroreceptor reflex before and after corticotrophin or prolonged chemoreceptor stimulation (see p. 220).

Our analysis of the minimum blood oxygen tension reached during the prolonged chemoreceptor stimulus and the size of the consequent delayed release, indicates that the threshold of the humoral response is around 50 torr (see figure 37). As our animals were anaesthetised and only the carotid chemoreceptors were subjected to the stimulus, the threshold may be higher in the intact animal. It is interesting to note that during periods of exercise, chronic bronchitics often have arterial

tensions well below 50 torr (King et. al.)

However, significant activation of the neuronal pathway to the adrenal medulla seemed to require oxygen tensions of around 20-30 torr and it appears that there are at least three mechanisms through which the adrenal glands may respond to hypoxia.

Firstly there is the humoral pathway, involving the anterior pituitary- adrenocortical axis.

Secondly, with more severe hypoxia there is the neuronal reflex which can come into play immediately in the emergency situation. (c.f. the body's response to a raised carbon dioxide tension via the central and peripheral chemoreceptors).

Finally, there is the possibility of a direct effect of hypoxia on the adrenal medulla but I have seen no convincing evidence that this is of any physiological importance.

APPENDICES

APPENDIX I.

A ROLE OF CIRCULATING NORADRENALINE

OUTLINE

In man, noradrenaline is the predominant catecholamine found in peripheral arterial and venous blood during rest and exercise. Over the last few years, it has been established that the main source of this noradrenaline is the peripheral adrenergic nerves and we wondered whether noradrenaline released from noradrenergic neurones has a role as a circulating hormone.

On bringing together the results from a number of studies concerned with exercise and ventilation, we found good evidence to suggest that during moderate exercise, arterial noradrenaline reaches concentrations where it is altering the sensitivity of the chemoreceptors to hypoxia.

We also thought that the metabolic actions of catecholamines might explain some observations made during a study on the respiratory response to exercise in patients with chronic respiratory failure.

However, before expanding these two themes, I shall attempt to put studies on peripheral plasma catecholamines in perspective.

INTRODUCTION

Sources of the Noradrenaline in the Blood

Assays of the catecholamine content of human adrenal glands which have been obtained from the post mortem room give a noradrenaline to adrenaline ratio of one to four.(see Von Euler 1956 p.111)

A few studies have been performed on samples taken from the adrenal vein during abdominal operations and these show that adrenaline is the predominant catecholamine secreted by the adrenal gland. Less than one quarter of the total catecholamine in peripheral arterial and venous blood is adrenaline and thus the noradrenaline of adrenal origin must represent under ten percent of that which is circulating. (eg. Seron, Stoppa, Plane & Bianchi 1958)

Furthermore, there is no detectable fall in noradrenaline excretion on denervation or removal of the adrenal glands while on the other hand, these procedures greatly reduce adrenaline excretion to levels where discrimination from the noradrenaline becomes unreliable. (eg. Von Euler, Franksson & Hellstrom 1954a-b)

N.B. To my knowledge, no one has attempted to set up a human isolated perfused adrenal gland preparation.

Sampling Sites

In any study on peripheral blood catecholamine levels, the choice of sampling site is of fundamental importance since there are efficient uptake mechanisms for both hormones.

Over 90% of an infusion of noradrenaline or adrenaline disappears on passing through peripheral vasculature such as skin or skeletal muscle.

This was shown by Celander and Mellander (1955) who compared the systemic effects of catecholamine infusions given either close arterially or intravenously. Vane's group have obtained similar results with studies on several vascular territories (see Vane 1969) and have also used their superfusion assay technique to demonstrate that adrenaline passes unchanged through the pulmonary circulation while 20-30% of noradrenaline is taken up by the lungs (Ginn and Vane 1968).

Whitby, Axelrod and Weil-Malherbe (1961) studied the uptake of an injection of radioactively labelled noradrenaline and adrenaline. Although they found a preferential concentration of noradrenaline compared to adrenaline in the lungs, very little of the radioactivity was detected in skeletal muscle. The distribution of the cardiac output probably determined the sites of uptake and much more activity was found in the heart, spleen and glandular tissue.

Physiological quantities of adrenaline and noradrenaline appear to be almost completely removed from the blood within a couple of circulations which is in agreement with the values determined for their half life of $2\frac{1}{2}$ minutes in man (Vendsalu 1960), and under twenty seconds in cats (Ferreira and Vane 1967).

Many workers cite peripheral venous concentrations as indices of "circulating" catecholamine levels. However, such a sample will be dominated by the noradrenaline released from the skin and muscle drained by the superficial vein (e.g. ante-cubital) subjected to venepuncture. Thus any changes detected only represent alterations in local sympathetic activity and possibly the effects of blood flow and metabolites on uptake, and

as such may be very interesting but do not reflect overall sympathetic activity nor act as an index of adrenal medullary discharge.

We were intending to investigate the role of circulating catecholamines as hormones and thus wanted to know their concentration in the arterial blood which perfuses the target organs.

A possible sampling site would have been the right atrium. However, as well as ethical and practical difficulties, there is good evidence that separate streams of blood extend through the right side of the heart as far as the branches of the pulmonary arteries (Claude Bernard 1865, Torrance and Ungar 1959). Furthermore, if pulmonary catecholamine uptake varies with cardiac output, mixed venous blood may not provide a reliable measure of arterial levels.

We therefore took arterial samples from a catheter in the brachial artery (the Department of Medicine was experienced with this procedure).

The results of other workers and the significance of the reported adrenaline levels

Mean Peripheral Plasma Levels of Adrenaline and Noradrenaline in Resting Subjects determined by the THI Method

	Adrenaline ng/ml (±SE)		Noradrenaline ng/ml (±SE)	
	Arterial	Venous	Arterial	Venous
Price & Price (1957)	0.10 ± 0.10	0.01 ± 0.07	0.20 ± 0.12	0.34 ± 0.15
Cohen and Goldenberg (1957)	-	0.06 ± 0.05	-	0.03 ± 0.07
Vendsalu (1960)	0.23 ± 0.02	0.07 ± 0.01	0.31 ± 0.02	0.40 ± 0.02
Valori, Renzini & Brunori (1970)	-	0.066 ± 0.013	-	0.174 ± 0.024
Häggendal (1963)				0.3 ± 0.1

Under most circumstances, Häggendal was unable to detect adrenaline in arterial and venous blood and says "... probably less than about 20% of the actual value quoted is likely to be adrenaline" (Häggendal, Hartley and Saltin 1970).

Holtzbauer and Vogt (1954) using bioassay were only able to state that adrenaline was less than 0.06 ng/ml in venous plasma.

However, the other groups mentioned in the table do give adrenaline values but one would be aware of the following points when considering such results:-

(a) These estimations are very near the limit of sensitivity of the trihydroxyindole technique

(b) The discrimination of this type of differential assay becomes unreliable when one of the pair of substances is in excess of 90% of the total (Gaddum 1959)

(c) As a consequence of skin and muscle uptake one would only expect to find a tenth of the already low arterial adrenaline concentration in peripheral venous samples.

The situation is different when there is a considerable adrenal discharge as is seen during insulin hypoglycaemia. Vendsalu (1960) published a graph showing a pronounced rise in peripheral venous adrenaline concentration following insulin administration with no change in the noradrenaline level. Häggendal (1963) also finds adrenaline in venous plasma after insulin administration as did Miller (1956 - using the ethylene diamine method), and Holzbauer and Vogt (1954 - using bio-assay). Thus it appears that when arterial adrenaline levels are sufficiently raised a measurable amount is found in the peripheral venous

blood. (Adrenaline is known to dilate skeletal muscle through-channels and this may enable a proportion to bypass the uptake mechanisms).

Although most reports of resting arterial adrenaline levels and venous levels during stress probably have some significance, I am very sceptical of some of the more recent clinical publications and especially those based on the double isotope derivative technique. - (I have already discussed the limitations of this method with regard to differential assay.)

Noradrenaline and alterations in the sensitivity to hypoxia

Asmussen and Nielson (1958) found that the respiratory minute volume is greater in hypoxic than in normoxic exercise by an amount which increases with the intensity of the work. Furthermore, a degree of hypoxia which would have no effect on an individual at rest leads to an increase in ventilation when the subject is exercising and this increase is a function of the work load.

It is well established that arterial catecholamine levels increase with exercise and this is supported by studies on venous blood and urinary excretion (see below).

Cunningham, Hay, Patrick and Lloyd (1963) showed that intravenous infusions of noradrenaline stimulated ventilation under hypoxia but not hyperoxic conditions.

We wondered whether the increase in arterial noradrenaline found during exercise could explain the potentiation of the ventilatory response to hypoxia.

From the data of Asmussen and Nielson one sees that an increase

in ventilation with hypoxia only becomes noticeable above a $\dot{V}_{O_2}$ of about $1\frac{1}{2}$ 1/min and at $2\frac{1}{2}$ 1/min the increase is 71%. (The rise in pH and fall in $PaCO_2$ resulting from the hyperventilation in these experiments would tend to decrease chemoreceptor responses to hypoxia).

Arterial noradrenaline rises with increasing $\dot{V}_{O_2}$ (Häggendal, Hartley and Saltin 1970) and this agrees with studies using venous plasma (Vendsalu 1960, Bannister and Griffiths 1972) and urinary output (see von Euler 1969). Significant adrenal discharge appears to occur only with prolonged or severe exercise (von Euler 1969).

With oxygen consumptions up to about 2 1/min, Häggendal et al (1970) found that arterial noradrenaline concentration rose gradually to just over 1 ng/ml. However, above this $\dot{V}_{O_2}$ the rise was much more steep and at 3 1/min the concentration was over 4 ng/ml.

It was also in the 2-3 1/min $\dot{V}_{O_2}$ range that the hypoxic and normoxic curves show a marked divergence on the minute volume against oxygen consumption graph of Asmussen and Nielson (1958).

The latter authors used 12.6% O_2 as a hypoxic stimulus which would give a PaO_2 of 38 torr (under normal atmospheric conditions). At a higher PaO_2 (around 45 torr) Cunningham et al (1963) found that an intravenous infusion of noradrenaline (10 µg/min) more than doubled ventilation.

We calculated that in the human subject, this rate of intravenous infusion would give an increase in arterial plasma noradrenaline of $2\frac{1}{2}$ - $3\frac{1}{2}$ ng/ml.

i.e. By assuming:-

Cardiac output = 5 1/min

Haemotrocit = 45%

20 - 30% uptake by the lungs

90% uptake by the tissues, e.g. skeletal muscle, liver etc.

A noradrenaline infusion of 10 µg/min is being mixed with a cardiac output of 5 1/min

Arterial Blood concentration = 2 ng/ml
Arterial Plasma concentration = 3.6 ng/ml

As 10% recirculates and 20-30% is removed by the lungs it seems reasonable to assume that the above circulated plasma concentration is attenuated by 20%.

Predicted plasma concentration of the infused noradrenaline= 3.0 ng/ml

Most users of the Trihydroxyindole catecholamine assay technique quote resting arterial plasma noradrenaline concentrations of 0.2-0.3 ng/ml (e.g. Vendsalu (1960), Price and Price (1957)).

Thus we would expect the steady state arterial noradrenaline concentration achieved in the experiments of Cunningham et al (1963) to be in the same range as Häggendal et al (1970) found at the higher work loads of up to 3 1/min.

Now, Cunningham's group found that this concentration of arterial noradrenaline resulted in a doubling of ventilation at arterial oxygen tensions of about 45 torr.

Thus the levels of between 2-4 ng/ml that would be expected at a $\dot{V}_{O_2}$ of 2½ 1/min (Häggandal et al 1970) might explain the 70% increase

in ventilation that Asmussen and Nielson (1958) observed in response to hypoxia at this work load.

The respiratory group at the Department of Medicine, Royal Infirmary, Edinburgh had investigated the respiratory responses of a group of patients in chronic respiratory failure to breathing air and a 30% oxygen mixture while at rest and exercising on the treadmill, (King, Cooke, Leitch and Flenley 1973). These patients were chronically hypoxic even when at rest (mean PaO_2 = 49 torr) and light exercise resulted in an even lower arterial oxygen tension (mean PaO_2 = 42 torr).

King et al (1973) made a number of interesting observations when they compared hypoxic exercise (i.e. when air breathing) with that done at an identical treadmill speed but with the hypoxia relieved by breathing 30% oxygen. (The order of air and 30% oxygen breathing was randomly allocated). During hypoxic exercise the following parameters were higher than when the patients were breathing 30% oxygen:

1. Minute volume
2. Carbon Dioxide output
3. Respiratory exchange ratio
4. Plasma lactate concentration

However, the lactate to pyruvate ratios were not significantly different and thus there appeared to be no change in the balance of aerobic to anaerobic respiration during the hypoxic period.

On the other hand when the patients were at rest none of the above four parameters was significantly affected by the change in gas mixtures.

It had been shown in a similar group of patients at rest that plasma catecholamines were two to three times normal levels, (Keller, Lohmann and Schüren 1971). These estimations were made on resting subjects and we thought it likely that exercise would further elevate catecholamine levels. This would then be a possible explanation for the potentiation of ventilation by hypoxia (i.e. the increased minute volume) while the patients of King et al (1973) were exercising.

Metabolic Effects

Catecholamine infusions have been shown by many workers to have metabolic effects such as raising plasma lactate and free fatty acid levels as well as increasing oxygen consumption (e.g. Nahas 1970). The combination of exercise with the hypoxic drive from the chemoreceptors may have lead to considerable sympathetic and adrenal medullary activation during the periods of hypoxic exercise. We wondered whether this might explain the changes in carbon dioxide output and plasma lactate concentration. One possibility was that the increase in plasma lactate would displace carbon dioxide from the bicarbonate buffer system. (In their discussion, King et al (1973) consider that any changes in carbon dioxide output due to the altered minute volume influencing the work of breathing or body carbon dioxide stores, would be too small to account for the discrepancy between the two types of exercise).

The Department of Medicine was planning a series of experiments to look at the effects of breathing 14% oxygen on normal subjects exercising at work loads of 1 and 1½ litres oxygen per minute. They were attempting to produce similar hypoxic conditions to those experienced by the patients while breathing air. Although we could not persuade them

to try a higher work load at which any effect of catecholamines would be more clear cut, (i.e. the 2-3 1/min range - see above discussion of Häggendal et al 1970 and Asmussen and Nielson 1958) we agreed to co-operate in the project and measure catecholamine concentrations in the arterial blood samples.

METHODS

Four healthy non-athletic men, aged 22-40 years, gave informed consent to the study. A catheter was inserted into the left brachial artery to enable arterial blood sampling for measurement of blood gas tensions, pH, lactate, pyruvate and catecholamines. We determined minute ventilation, oxygen uptake and carbon dioxide output and continuously recorded end tidal oxygen and carbon dioxide tensions using a Varian M3 mass spectrometer. All measurements were made both at rest, when standing and during the 7th, 23rd and 26th minute of steady state treadmill walking. Two differential treadmill speed settings were employed, both when breathing 14% oxygen (balance nitrogen) and when breathing air. Each subject walked for four separate 30 minute periods in the day, with intervals of at least 30 minutes rest. The subject did not know what gas he was breathing.

The various respiratory and arterial blood parameters (except catecholamines) were determined in the Department of Medicine. The analytic methods and apparatus are described in the publication by King et al (1973).

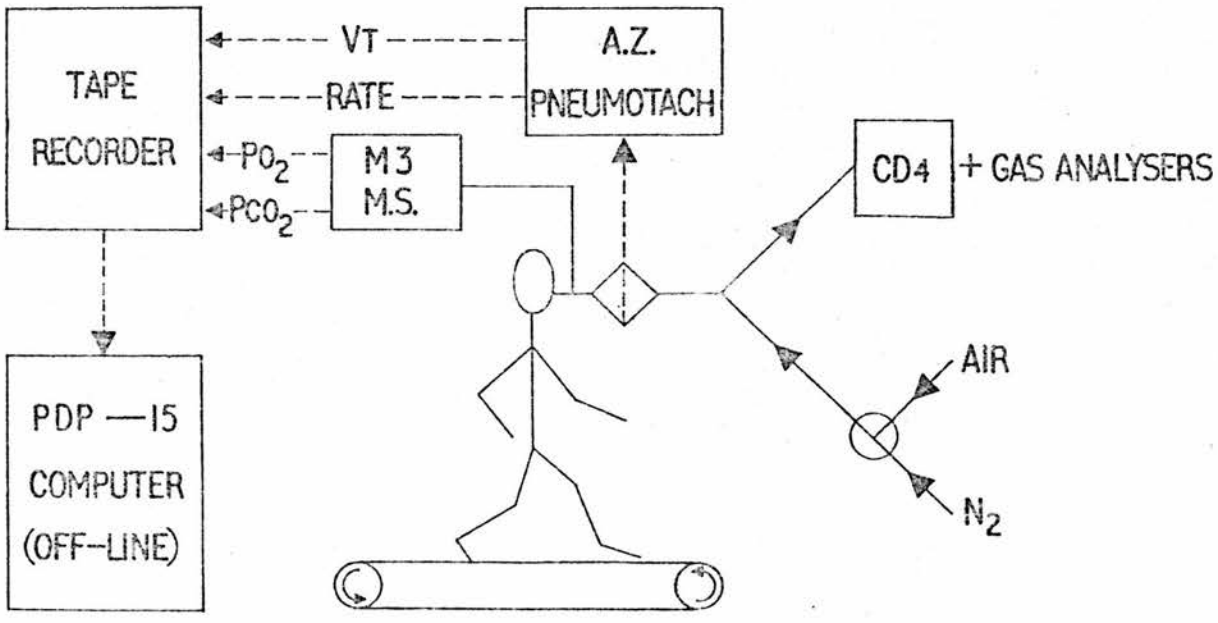


FIGURE 46.

Catecholamine Assay

We used the method which I have described for the assay of p129
catecholamines in adrenal venous blood. The catecholamine concentration in peripheral arterial or venous plasma taken from the resting subject, is in the range 0.2 - 0.4 ng/ml. We therefore expected our 10 ml blood samples to contain 1-5 ng of catecholamine and thus made the following modifications to our standard method in order to increase sensitivity.

1. Cleanliness

The greatest of care was taken when washing glassware or handling the columns and all the reagents were freshly prepared (i.e. I did it all myself).

This was necessary in order to:-

(a) Reduce the blank value to a minimum

(b) Reduce the scatter peak so that the fluorimeter slits could be widened.

2. Aminco-Bowman Spectrophotofluorometer

Wider slits were used:-

Excitation: 4 mm

Emission: 5 mm

3. Concentration Procedure

The catecholamine-containing eluate (4 mls of IN HCl) from the columns was evaporated to dryness under vacuum at 30°C - by means of an Evapomix.

Under these conditions the hydrochloric acid looses water until about 8N when both HCl and water are lost together.

The concentration of catecholamines by evaporation to dryness under vacuum in acid conditions had been used by Vogt (1952). As I mentioned earlier, catecholamines are very stable under acid conditions especially when no oxidising agents (eg. oxygen) are present.

We found that providing the temperature was kept below 35°C which necessitated great care with the vacuum seals, the recovery of this stage was always over 90%.

I spent some time trying to improve on this figure and examined the effect:

1. Polyethylene glycol - addition of 20 µl prior to evaporation
2. Ethanol (used in Vogt's technique) - addition of 1 ml prior to evaporation
3. Neutralisation prior to evaporation
4. Evaporation down to 0.5 ml instead of to dryness - followed by neutralisation

These modifications did not improve the recovery and only made the technique more tedious as well as raising the blank values. The small catecholamine loss may have been due to adsorption onto the glass or due to spray formation during evaporation.

4. Fluorimetry

We were able to use the same centrifuge tube for collecting the eluate, evaporation and fluorimetry.

Immediately the vacuum had been released the following was done to each of the samples:-

(a) 200 ul of 1M phosphate buffer (pH 6.5) added. We used 1M buffer instead of 0.1M in case any hydrogen chloride remained in the tube (the fluorimetry was unaffected by the use of more concentrated buffer).

(b) 1.4 ml water added.

(c) Solution "whirlymixed" for at least 1 minute - to ensure that all the catecholamine was dissolved.

(d) pH tested - if less than pH 6.5 (which very rarely happened), it was neutralised by dropwise addition of potassium bicarbonate (IN).

(e) 0.8 ml of the sample was transferred to another centrifuge tube (i.e. sample halved).

(f) 20 ul $CuCl_2$ was added to each tube.

(g) Our standard fluorimetric procedure as previously described was performed with one tube of each pair becoming the faded blank. p139

(h) The photomultiplier of the spectophotofluorimeter was switched to maximum gain and which resulted in some "random noise" on the traces and so we scanned each sample twice.

Comment on the Assay

1. It concentrated the sample $3\frac{1}{2}$ times more than the technique used for adrenal venous samples.
2. Duplicate estimations could not be performed (however, we had found that there was little variation between duplicates when assaying adrenal venous samples).
3. The fluorescent spectra of the arterial plasma samples showed the characteristic "double peak" of catecholamines.
4. At the wavelengths used for the differential assay the fluorescent spectra of the resting arterial plasma samples was 2-3 times the faded blank value.

5. Recovery experiments - We obtained a recovery of 84 ± 6% (S.D. n = 8) when 10 ng of noradrenaline was added to 10 mls of plasma.

6. I could never detect any adrenaline and assumed that it was less than 15% of the total catecholamine content in a similar fashion to Häggendal. (Häggendal et al 1970).

7. This is not the ideal assay for peripheral plasma catecholamines and I am well aware of its limitations. However, it was the only one we had available and as I have already discussed, no other chemical method appears to be any better (especially with regard to <u>differentiation</u> between noradrenaline and adrenaline). p120

RESULTS

The arterial blood gas tensions, pH and plasma noradrenaline concentrations, the minute ventilation ($\dot{V}_E$) and oxygen consumption ($\dot{V}_{O_2}$) for each period while standing and the mean values for each exercise period are shown in the table. p 264-5

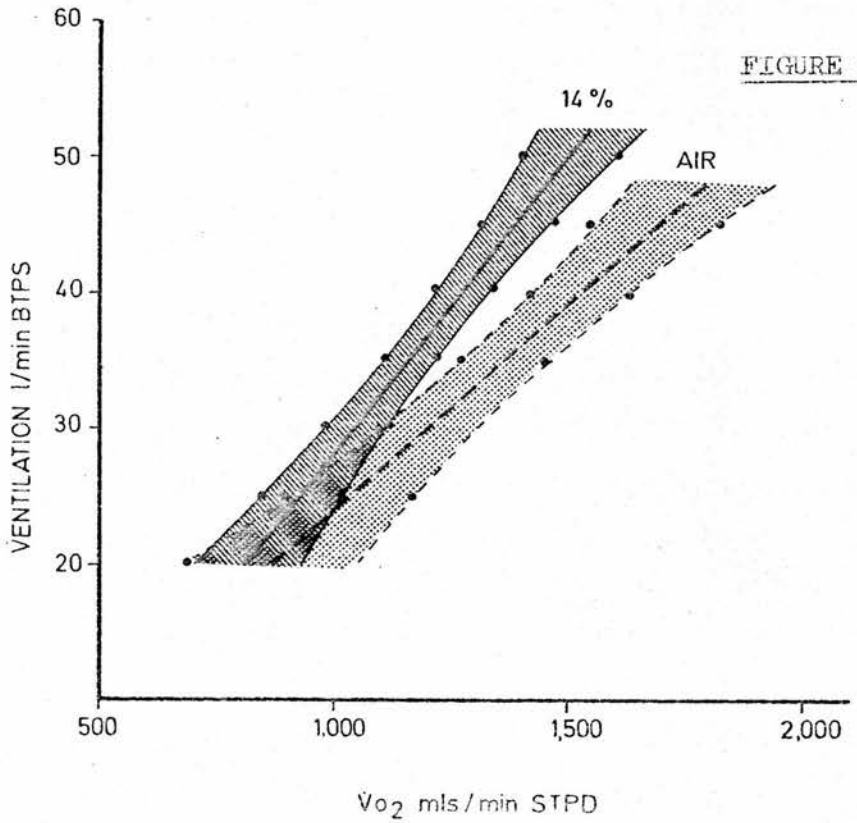
Ventilation

At rest the minute ventilation was not consistently altered by the breathing of 14% oxygen.

In each subject during exercise the minute ventilation was greater at both levels of exercise when breathing 14% oxygen than when breathing air.

The regression equations for the $\dot{V}_E$ - $\dot{V}_{O_2}$ relationships during hypoxia (14% O_2) and normoxia are plotted in the following graph:

FIGURE 47.



For the hypoxic line:-

$$\dot{V}_E = (0.032 \pm 0.003)\dot{V}_{O_2} - (2.03 \pm 4.26)$$

Correlation coefficient = 0.91

For the normoxic line:-

$$\dot{V}_E = (0.020 \pm 0.004)\dot{V}_{O_2} + (5.15 \pm 4.26)$$

Correlation coefficient = 0.84

The 95% confidence limits of these lines overlap when $\dot{V}_{O_2}$ is less than 1100 ml/min but are clearly separated when $\dot{V}_{O_2}$ is 1500 ml/min showing $\dot{V}_E$ is significantly greater during hypoxia at the higher exercise levels.

The mean arterial oxygen tensions during the two hypoxic exercise periods were similar but one subject hyperventilated during hypoxia when walking at 2.0 m/s. He achieved the highest minute ventilation and arterial noradrenaline concentrations, had a respiratory exchange ratio of 1.3 but did not have the highest oxygen uptake. He also hyperventilated during hypoxia when standing and his respiratory exchange ratio was then 1.34.

Catecholamines

NORADRENALINE - When the subjects were standing, the plasma noradrenaline concentrations were consistently greater when breathing 14% oxygen than when breathing air but the differences were not statistically significant ($P < 0.1$). Walking at 1.5 m/s while breathing 14% oxygen or air did not significantly elevate plasma noradrenaline but plasma noradrenaline was significantly elevated ($P < 0.01$) when subjects walked at 2.0 m/s breathing 14% oxygen. Normoxic walking at 2.0 m/s did not significantly elevate plasma noradrenaline ($P < 0.1$).

ADRENALINE - The adrenaline concentrations did not appear to exceed 15% of the total catecholamines in any of our experiments and so remained undetectable at rest and during exercise.

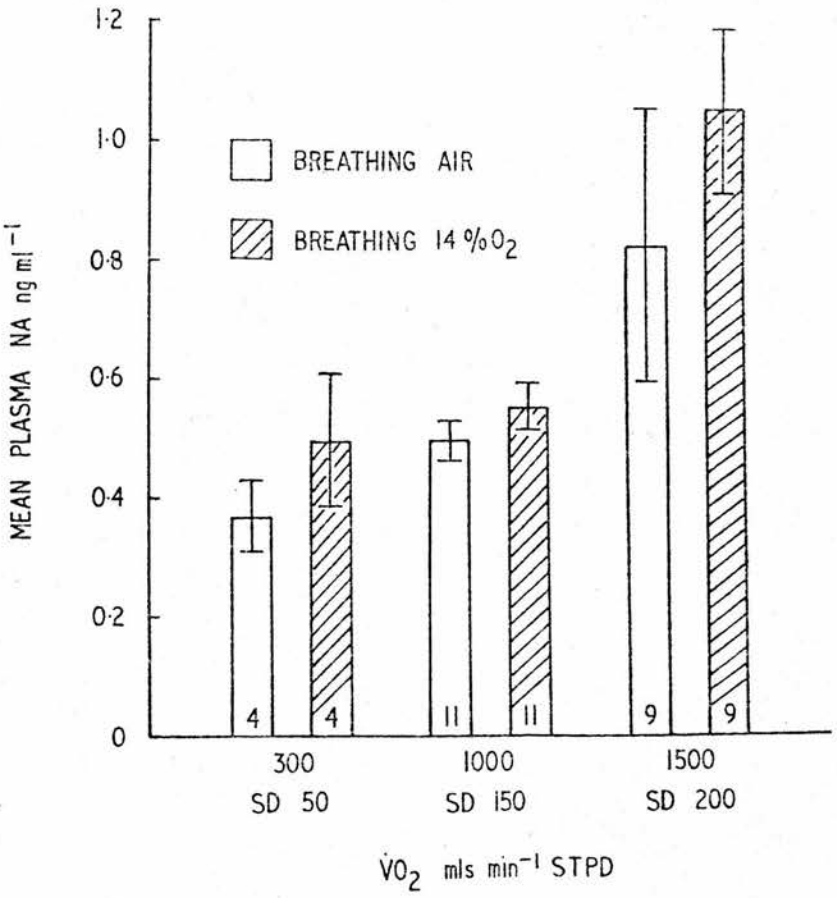


FIGURE 48.

Metabolic Indices

For interest, I have plotted the following graphs:-

1. R.Q. against plasma Noradrenaline (Fig. 49a) p 261

All the respiratory quotients and their corresponding plasma noradrenaline values have been used. There appears to be a linear relation between the R.Q. and the plasma noradrenaline concentration.

2. Increase in lactate against increase in pyruvate (ratios) (Fig. 49b)

The ratio of the plasma lactate while breathing the hypoxic gas mixture to that while on air is plotted against the corresponding ratio for the change in pyruvate with hypoxia.

The points fall on the 45° line and thus it appears that there was no consistent change in the proportion of aerobic to anaerobic metabolism on altering the composition of the inhaled gas mixture.

3. Plasma lactate against plasma noradrenaline (Fig. 50a) p262

Here I have examined the effect of the change in the gas mixture on the plasma concentration of lactate and noradrenaline. Different symbols have been used for the two work loads and the arrows point to the values measured during hypoxia.

At the higher work load, the period of hypoxia was accompanied by a consistent elevation of the plasma concentrations of both lactate and noradrenaline.

However, at the lower work load, the switch to hypoxia did not appear to be associated with any consistent change in either of these plasma values.

4. Increase with hypoxia in the plasma lactate against that in the plasma noradrenaline (Fig. 50b)

The elevations in the plasma concentrations of lactate and noradrenaline which were seen during hypoxia have been plotted against one another and the arrows now show the increase in the work load.

The elevations of both the plasma lactate and the plasma noradrenaline were consistently greater at the higher work load.

5. Excess carbon dioxide output during hypoxia against the increase in plasma noradrenaline with hypoxia (Fig. 51a) p263

There appeared to be an increase in carbon dioxide production while the exercising subjects were breathing the 14% oxygen mixture.

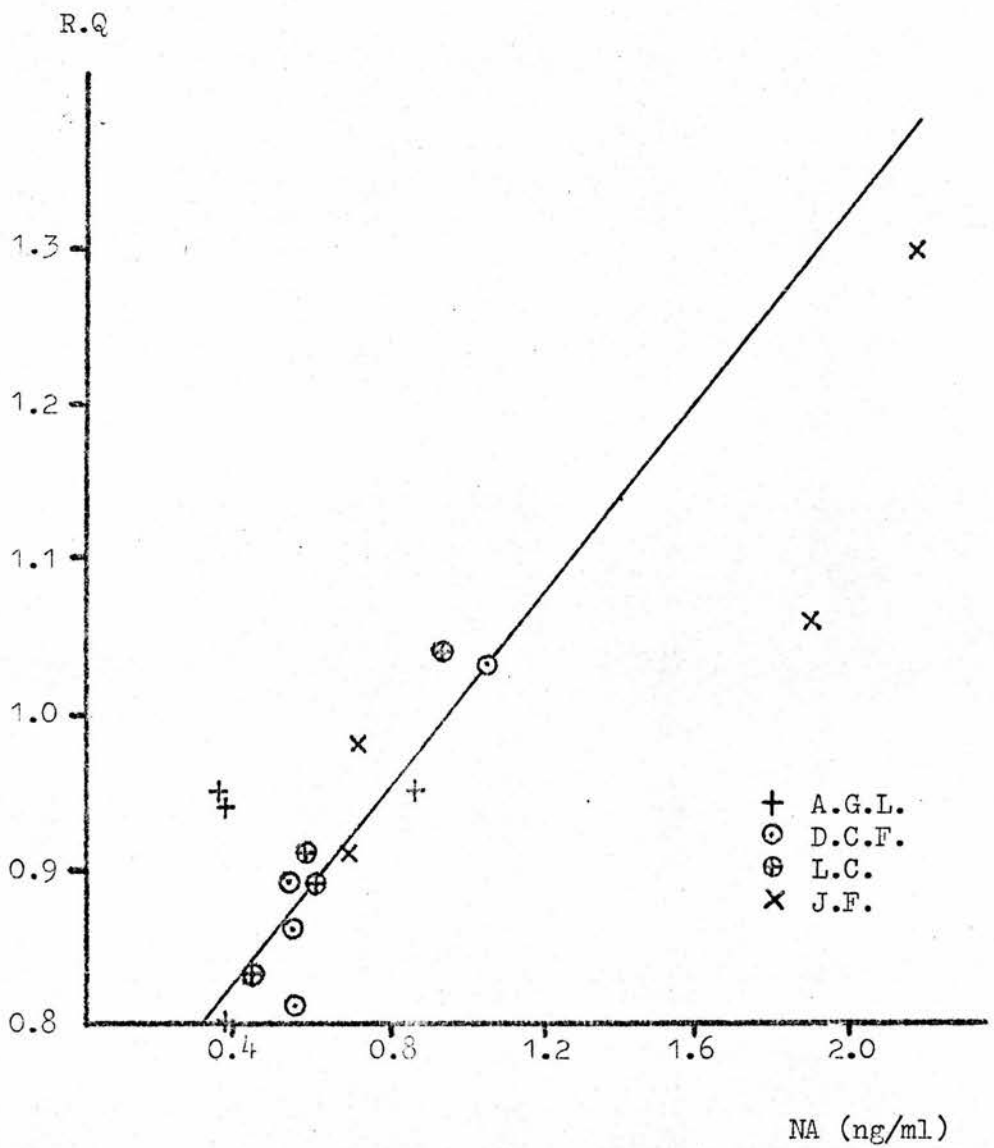
We use the term excess carbon dioxide output to represent the difference between the observed and the expected carbon dioxide outputs during hypoxia. The expected $\dot{V}_{CO_2}$ was the product of the $\dot{V}_{O_2}$ measured during hypoxia and the R.Q. calculated from the values obtained during the normoxic period at the same work load.

The excess carbon dioxide output has been plotted against the difference between the plasma noradrenaline concentration during hypoxia and that during normoxia.

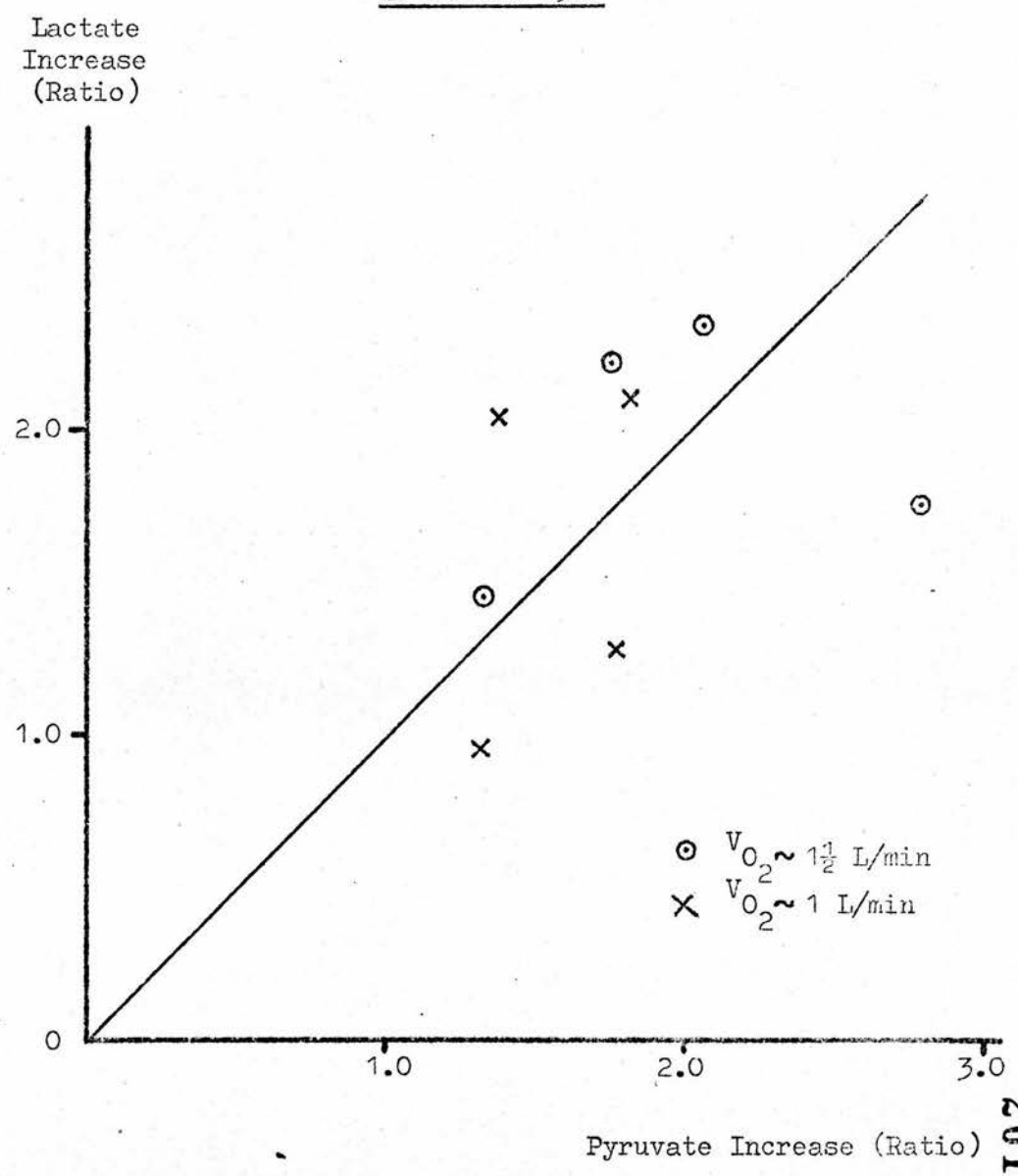
6. Excess carbon dioxide output against the increase in plasma lactate (Fig. 51

The excess carbon dioxide output values used in the previous graph are now plotted against the increase in the plasma concentration of lactate seen during hypoxia.

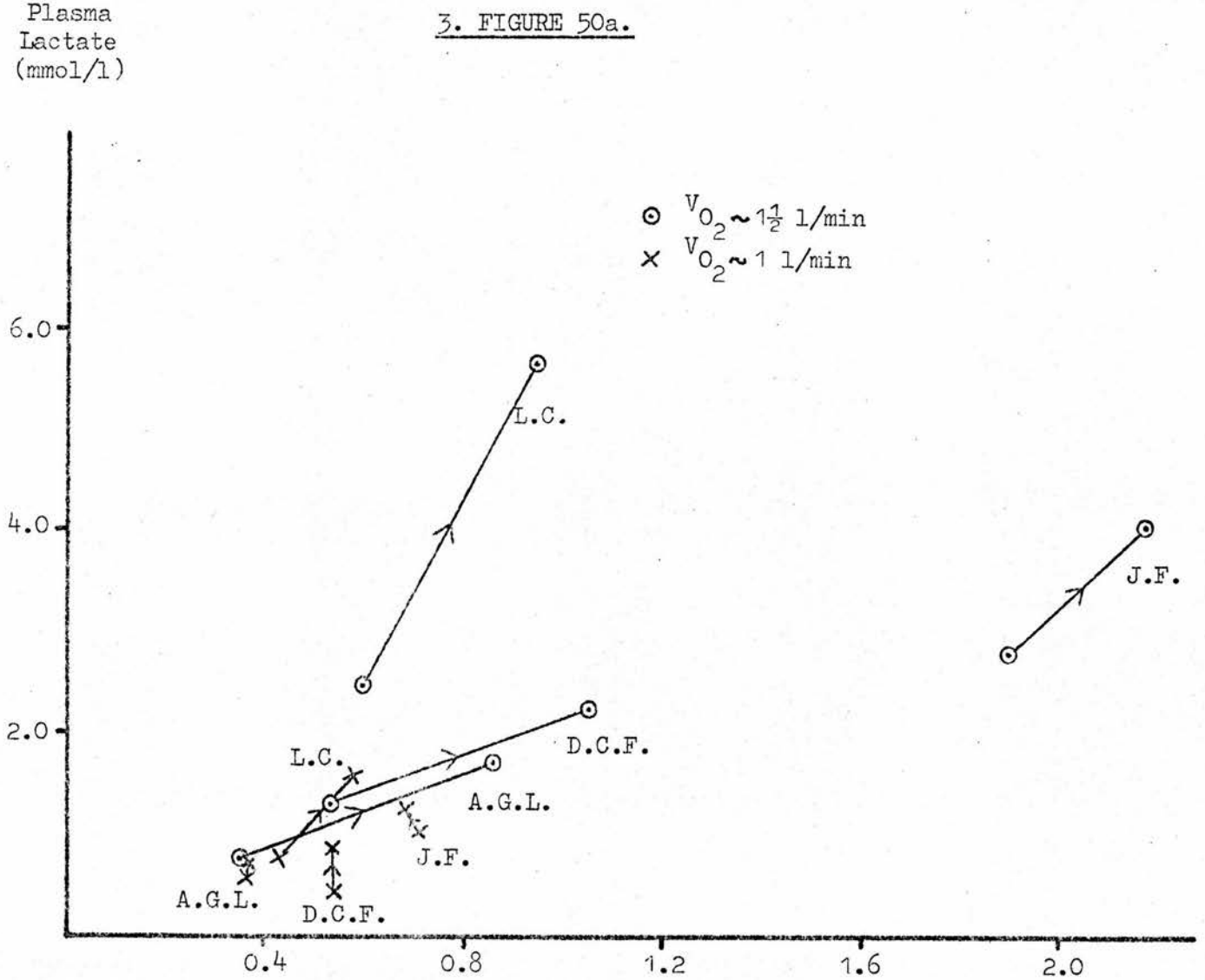
1. FIGURE 49a.



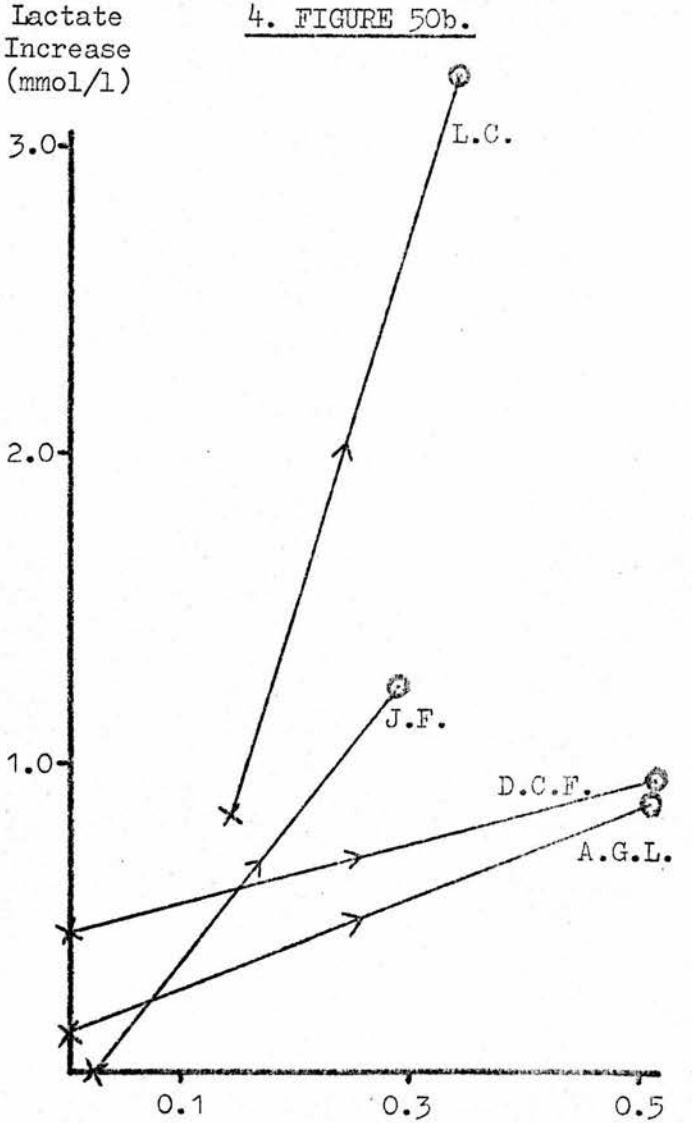
2. FIGURE 49b.



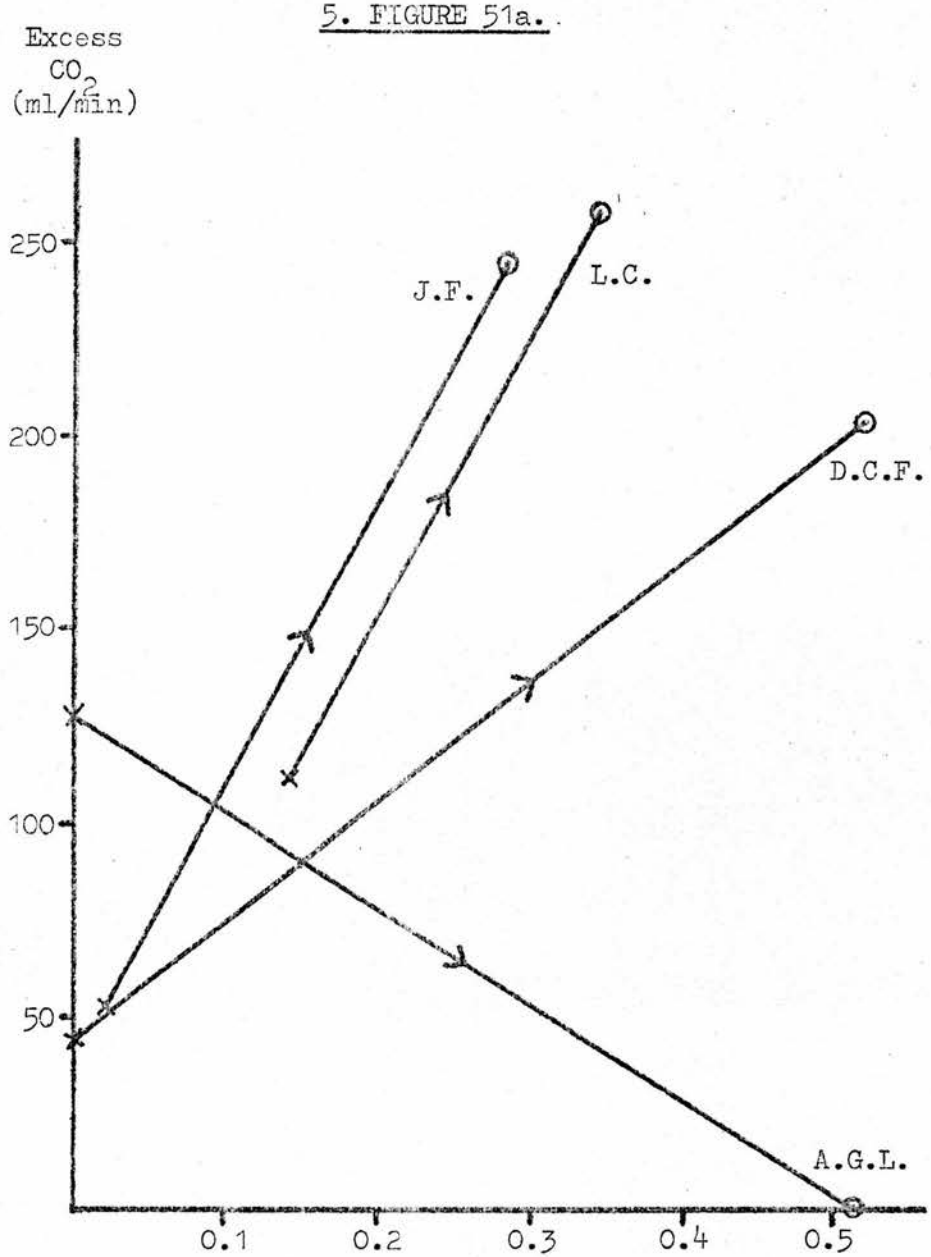
3. FIGURE 50a.



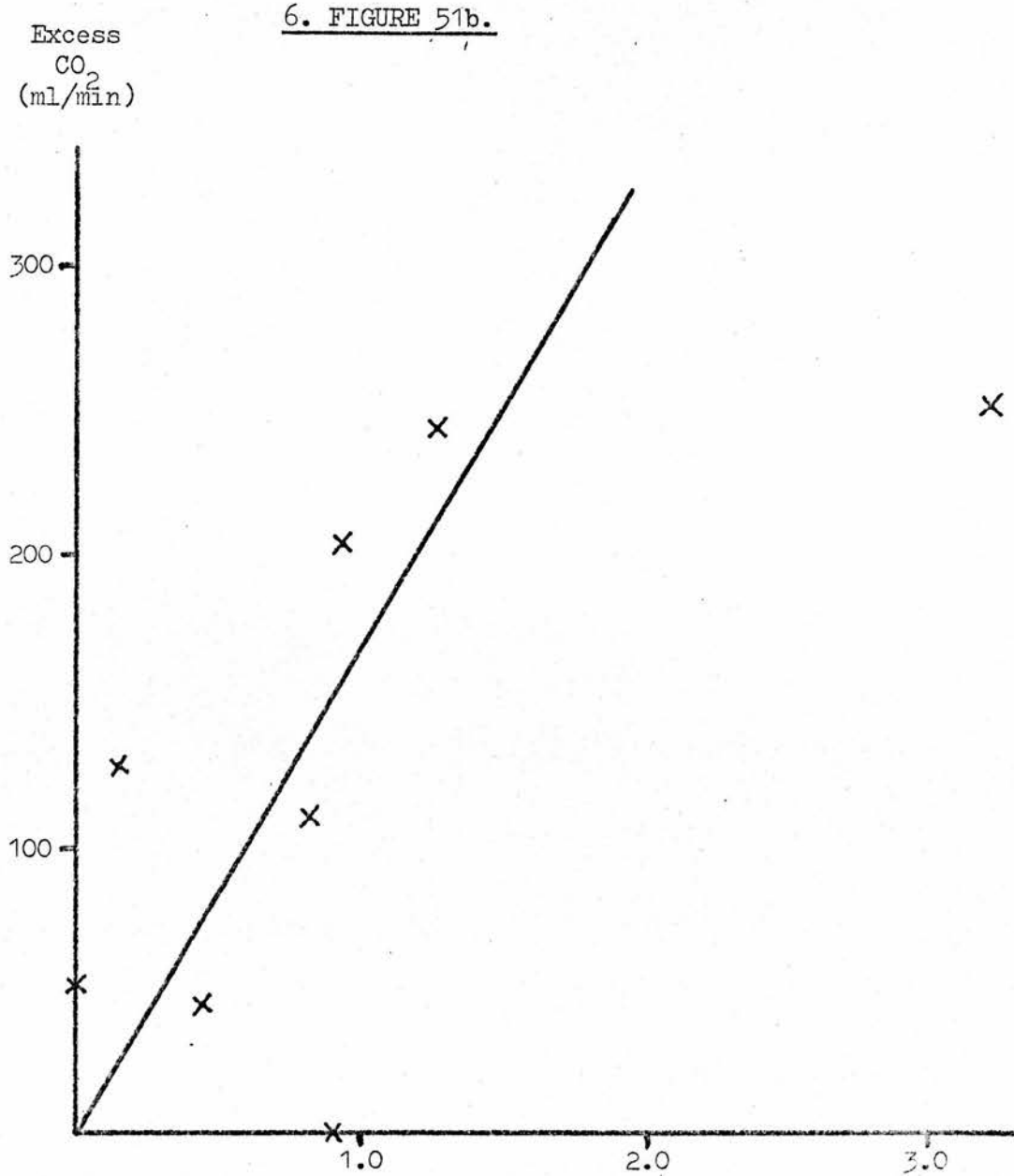
4. FIGURE 50b.



5. FIGURE 51a.



6. FIGURE 51b.



MEAN RESPIRATORY AND ARTERIAL BLOOD VALUES AT REST AND DURING TREADMILL WALKING IN FOUR NORMAL MALES BREATHING AIR OR 14% OXYGEN

	F_{IO_2} %	$\dot{V}_{O_2}$ ml/min STPD	$\dot{V}_{CO_2}$ ml/min STPD	$\dot{V}_E$ L/min BTPS	R	H.R/min	NA ng/ml plasma	ARTERIAL BLOOD P_{O_2} torr	ARTERIAL BLOOD P_{CO_2} torr	ARTERIAL BLOOD pH	ARTERIAL BLOOD Lactate mmol/L	ARTERIAL BLOOD Pyruvate mmol/L
1. A.G.L	14	238*	188	8.6	0.79	72	0.2	53	35	7.40	0.6	0.12
Ht. 182 cm	21	330*	234	9.9	0.71	84	0.2	99	39	7.40	1.6	0.18
Wt 66.5 kg	14	946**	871	28.4	0.94	99	0.4	50	39	7.40	0.7	0.10
S.A. 1.85 m^2	21	1004**	790	26.2	0.78	90	0.4	100	38	7.40	0.5	0.60
	14	1427***	1354	43.9	0.95	123	0.9	46	39	7.42	1.7	0.14
	21	1336***	1272	38.7	0.95	108	0.3	101	38	7.43	0.7	0.09
2. D.C.F	14	278*	228	10.7	0.82	71	0.5	60	35	7.43	0.4	0.04
Ht. 175 cm	21	353*	282	12.9	0.80	111	0.4	87	37	7.44	0.6	0.08
Wt. 76 kg	14	983**	846	28.4	0.86	107	0.5	48	34	7.42	0.9	0.05
S.A. 1.95 m^2	21	1013**	827	27.5	0.81	102	0.5	100	37	7.43	0.4	0.04
	14	1387***	1438	47.3	1.03	141	1.0	50	35	7.44	2.2	0.09
	21	1492***	1328	42.8	0.89	131	0.5	94	37	7.44	1.3	0.03

NB: F_{IO_2} = Inspired oxygen concentration; $\dot{V}_{O_2}$ = Oxygen consumption; $\dot{V}_{CO_2}$ = Carbon dioxide consumption; $\dot{V}_E$ = Expired minute ventilation; R = Respiratory Exchange ratio; H.R = Heart rate; NA = Noradrenaline concentration; P_{O_2} and P_{CO_2} = Oxygen and Carbon dioxide tensions; S.A. = Body surface area

Continued over

Continued

	F_{IO_2} %	$\dot{V}_{O_2}$ ml/min STPD	$\dot{V}_{CO_2}$ ml/min STPD	$\dot{V}_E$ L/min BTPS	R	H.R/min	NA (plasma) ng/ml	ARTERIAL BLOOD P_{O_2} torr	P_{CO_2} torr	pH	Lactate mmol/L	Pyruvate mmol/L
3. L.C.	14	373*	325	11.5	0.87	75	0.5	57	41	7.39	0.7	0.05
Ht. 177 cm	21	408*	306	11.2	0.75	87	0.4	99	40	7.41	1.5	0.09
Wt. 82.5 kg	14	1291**	1185	32.8	0.91	108	0.6	49	40	7.40	1.6	0.08
S.A. 1.98 m^2	21	1275**	1185	28.5	0.83	99	0.4	96	42	7.41	0.7	0.05
	14	1677***	1751	48.8	1.04	154	0.9	47	47	7.40	5.6	0.17
	21	1973***	1758	40.9	0.89	147	0.6	85	43	7.42	2.4	0.08
4. J.F.	14	237*	318	10.0	1.34	102	0.7	63	33	7.46	1.3	0.06
Ht. 180 cm	21	293*	255	9.6	0.87	129	0.6	97	36	7.46	1.3	0.06
Wt. 56 kg	14	827**	807	22.1	0.98	128	0.7	50	36	7.44	1.2	0.08
S.A. 1.71 m^2	21	868**	793	21.6	0.91	126	0.7	99	38	7.44	1.2	0.06
	14	1167***	1483	48.2	1.30	184	2.2	62	31	7.45	4.0	0.20
	21	1536***	1631	47.7	1.06	188	1.9	99	34	7.46	2.8	0.15

Speed	Number of determinations
* 0 m/s	(n = 1)
** 1.5 m/s	(n = 2)
*** 2 m/s	(n = 3)

DISCUSSION

The changes in the various metabolic indices which were seen on altering the composition of the inspired gas mixture while the subjects were exercising at the higher work load, are interesting and deserve further investigation. One can speculate that the increased plasma catecholamine concentration has a metabolic effect and possibly the excess carbon dioxide output is a consequence of the addition of more lactic acid to the blood.

Our results are compatible with the arguments put forward in this appendix's introduction that increased concentrations of arterial plasma noradrenaline contributed to the hypoxic potentiation of the respiratory response to moderate exercise. We found that during hypoxic exercise at a $\dot{V}_{O_2}$ of about 1½ l/min, the noradrenaline concentrations were double those when the $\dot{V}_{O_2}$ was around 1 l/min and it was only at the higher treadmill speed that the $\dot{V}_E$ was significantly greater during the hypoxia compared to normoxic conditions.

At gas tensions very similar to those prevailing during our periods of hypoxic exercise Patrick (1964) found that a noradrenaline infusion of 5 ug/min increased ventilation by about 50%. We estimate (see p.247) that this infusion rate would have resulted in a rise in arterial catecholamine concentration of between 1 and 1½ ng/ml which is twice that observed in our experiments.

Thus, in our experiments, the elevated arterial noradrenaline concentration may have contributed to the 25% increase in ventilation seen in response to hypoxia when our subjects were exercising at a $\dot{V}_{O_2}$ of 1.5 l/min.

APPENDIX II.

FURTHER STUDIES ON PERIPHERAL PLASMA CATECHOLAMINE ASSAY

I have described in Appendix I on our studies in Man how p 252
the method for the quantitative estimation of catecholamines in adrenal venous blood was applied to peripheral arterial plasma.

The main problem we had to overcome when assaying these samples was the large final volume of eluate (4 mls) which would contain only a few nanograms of catecholamine. The "Evapomix" successfully concentrated the catecholamines and the overall recovery of the technique was above 80% (e.g. 84 $\pm$ 6%). However, the technique is unsuitable for routine use because of the care required in using the "Evapomix". We also tried freeze drying the samples prior to fluorimetry. This was satisfactory, giving recoveries of over 90% for this step, but depended on the availability of a freeze drier.

In looking for an alternative technique for the estimation of adrenaline and noradrenaline in peripheral blood, we had two main objectives:

1. To use as small an eluting volume as possible so as to achieve maximum concentration of catecholamines.
2. To reduce the blank value.

We were also concerned with finding a technique which could be performed using our existing apparatus and was simple enough for routine use by a technician.

We first tried to obtain more concentrated eluates by:-

(a) Eluting with stronger acid

(b) Reducing the length of the columns

(a) Elution with stronger acid

Using catecholamine standards, we found that 1 ml of 4N HC1 would completely elute catecholamines from 2 cm columns (Amberlite CG-120), yielding recoveries of over 90%.

On applying this technique directly to peripheral plasma, recognisable catecholamine fluorescence curves were obtained but the blanks were very high and variable and some of the eluates were cloudy (this could not be completely removed by centrifugation).

(b) Reduced volume of resin

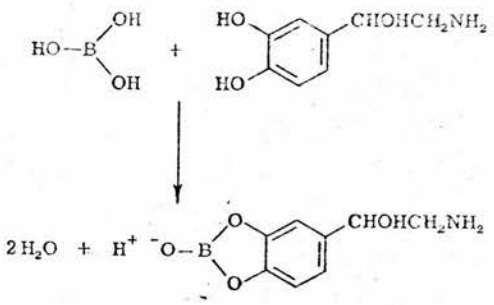
Using pure standard solutions, we confirmed that only 2mls of 1N HC1 are required to completely elute the catecholamines from 1cm columns. However, the ionic content of 10 mls of plasma is sufficient to wash 40% of the catecholamine off this size of column, hence this approach to the reduction in elution volume is unsatisfactory.

The use of Boric Acid for Elution

Trauther and Nesser (1952) reported that noradrenaline and adrenaline form complexes with boric acid. This property is shared with dopamine but not with their ortho-methylated derivatives. The complexes are highly stable at pH 7-8 and can safely be stored for several days under refrigeration (Wright 1958).

Wright (1958) was the first to use borate complexes in the extraction of catecholamines and more recently methods have been described by Mattock and Wilson (1966) and Sandhu and Freed 1968).

FORMATION OF BORATE - NORADRENALINE COMPLEX



Initially we attempted to elute catecholamines from our 2 cm columns of CG-120 resin by using a 2/3 Molar solution of boric acid. However, this could not be achieved in a sufficiently small volume and we were unable to further increase the concentration of the boric solution as, even at this strength, it had to be stirred and heated continuously for several minutes before it could be completely dissolved.

On theoretical grounds, we thought that increasing the pH of the eluting fluid would favour the elution of the borate-catecholamine complexes and thus we studied the effect of adding sodium hydroxide to the boric acid.

At a pH of 6.5 we found that 10 mls of the borate solution was required to elute 95% of the catecholamine that had been loaded onto the columns and the recovery after elution with 5 mls of the solution was only 70%. However, at a pH of 8.0, elution with 5 mls of borate solution gave a recovery of 91%.

We felt it was undesirable to further increase the alkalinity of the borate solution as catecholamines are known to oxidise more readily in alkaline conditions.

So far we had failed to achieve sufficient concentration of catecholamines in our final eluate and next turned our attention to the use of boric acid to elute from weak cation exchange resins, e.g. Amberlite CG-50.

The use of Amberlite CG-50

Amberlite CG-50 is a carboxylic acid, weak cation exchange resin. We used the resin in the sodium/potassium form, after the same preparation procedure to that previously described for the sulphonic acid resin. p136 ,

Catecholamines were eluted from the resin with 2/3 molar boric acid following a single rinse with distilled water. The eluates could be used directly in the trihydroxyindole fluorimetry process, after adjusting them to pH 6.5 with a small quantity of 1N HC1.

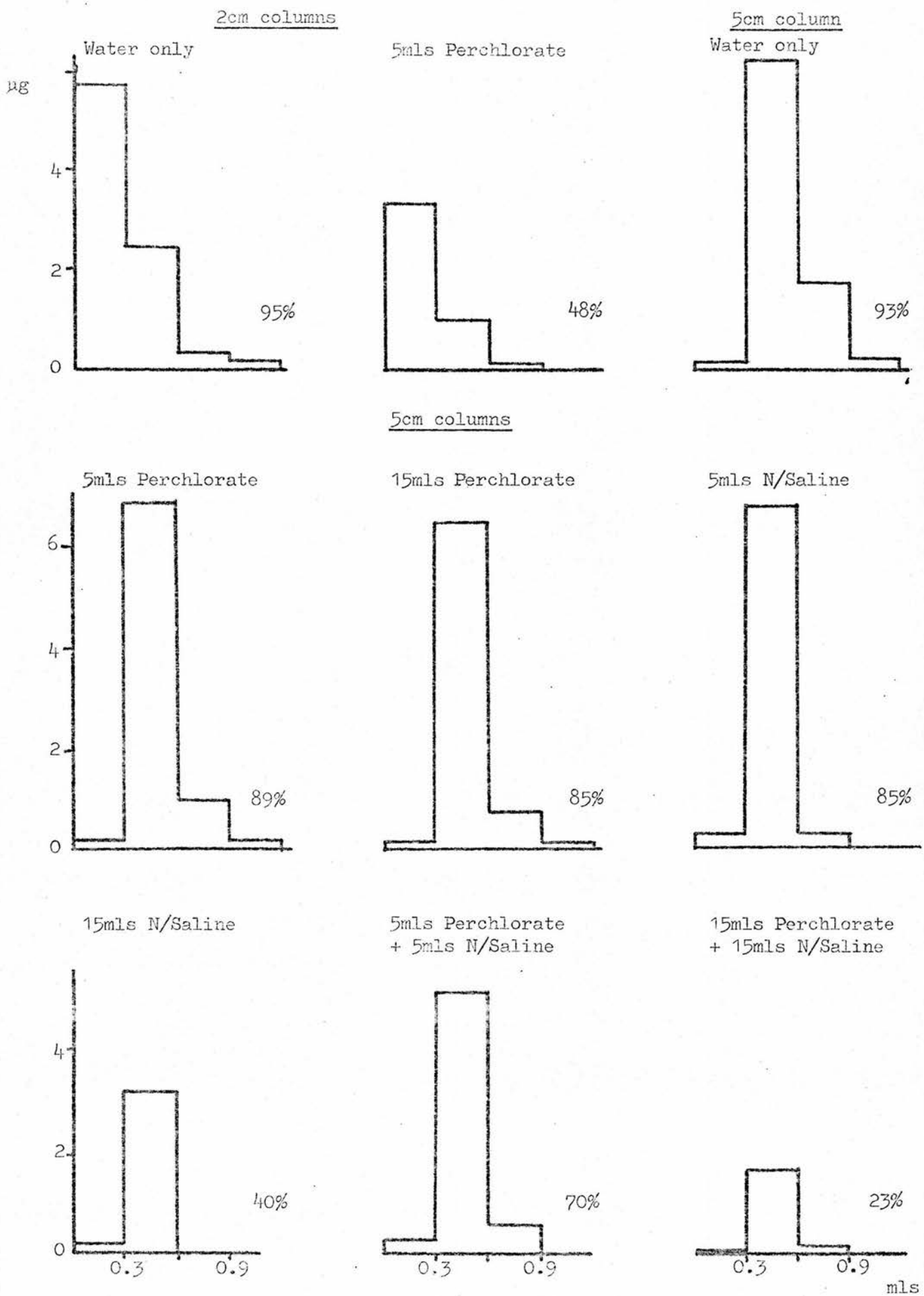
The initial work was done using solutions of pure catecholamine standards (100 ng) and columns of 2 cm length. Complete elution could be performed with 0.6 mls of boric acid and recoveries were found to be over 90%. When the ionic content of the catecholamine solution was increased by the addition of a few mls. of neutralised perchloric acid solution (after centrifugation to remove the precipitate) the recovery was reduced to about 50%.

So as to overcome this loss the columns were lengthened to 5 cm. These still had a satisfactory flow rate and elution was completed with 1 ml of boric acid giving recoveries of over 90%.

Bearing in mind that plasma has a relatively high ionic content even before the addition of perchlorate ions, the recovery experiments were repeated in the presence of 0.9% saline with and without neutralised

FIGURE 52.

EFFECT OF COLUMN LENGTH AND PRESENCE OF INORGANIC IONS (SALINE AND PERCHLORATE) ON RECOVERY OF CATECHOLAMINE STANDARDS FROM COLUMNS OF AMBERLITE CG-50.
Elution performed with Boric Acid (⅜M) in 0.3ml fractions.



perchlorate. The elution profiles and recoveries obtained are illustrated in Figure 52 and it can be seen that the presence of all these ions leads to very high losses from the resin.

We supported these observations by adding small amounts of catecholamines to plasma prior to which protein precipitation had been performed using perchloric acid, followed by neutralisation and centrifugation. The solutions were loaded onto the 5 cm columns and eluted with 2/3 M boric acid.

Average recovery from 5 mls plasma was 73% (n = 6) and from 10 mls plasma it was even lower (60%). This was unsatisfactory as we intended to subject the columns to the ionic load contained in 20 ml blood samples i.e. (10 mls plasma); although not surprising, as we had feared that the weak acid resin would be unable to cope with a high ionic load.

A Two-column Extraction Technique

Renzini, Brunori and Valcri (1970) described a technique in which they overcame the problem of plasma's relatively high ionic content. They performed an initial extraction of the catecholamines using alumina and eluting with 0.05N perchloric acid. This eluate was then passed through an ion exchange resin of carboxylic acid form. Final elution was performed with 0.5 mls of boric acid. They only dealt with initial plasma volumes of 5-10 mls because a high ionic load also affected the recovery from alumina. They used micro-curvettes for the fluorimetry so as to increase the final concentration of the fluorophors.

For reasons previously discussed we did not want to use p135
alumina. However, we found that elution of catecholamines from 2 cm amberlite CG-120 could be performed with 4 mls of 1N perchloric acid. Recoveries were similar to those obtained with 1N hydrochloric acid i.e. (over 90%). The elution profiles are compared in Figure 53.
The strong acid resin can cope with a higher solute load and no losses were found in the presence of 10 mls saline although 20 mls of saline resulted in a reduction in recovery by almost 30%.

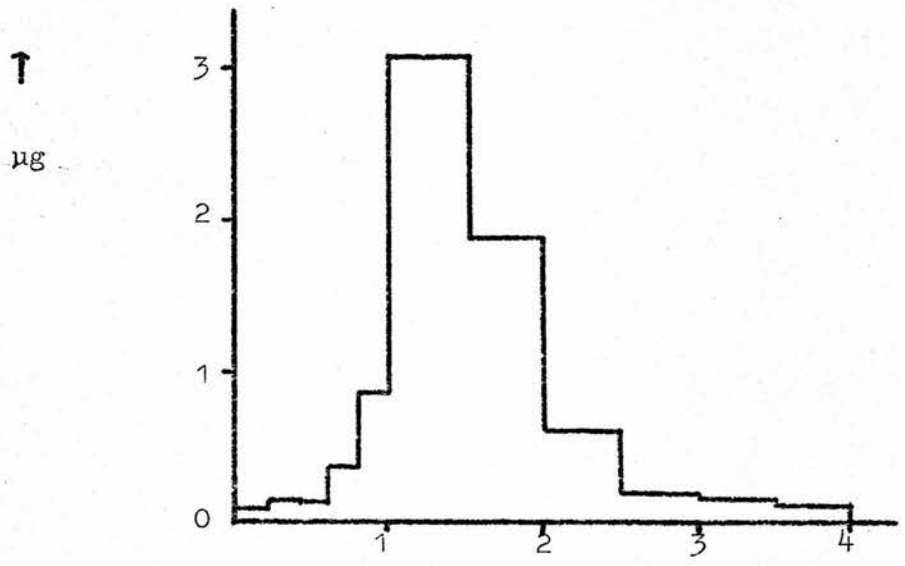
For the second stage of the procedure, the perchloric eluates were neutralised, frozen and centrifuged in order to remove the perchlorations before loading the supernatant onto 5 cm columns of amberlite CG-50. The columns were rinsed with 10 mls of distilled water before performing the final elution with 1 ml of 2/3 M boric acid.

Recovery using pure standard solutions was found to be over 85% and equivalent values were obtained when this was repeated using standards added to 10 mls of prepared plasma.

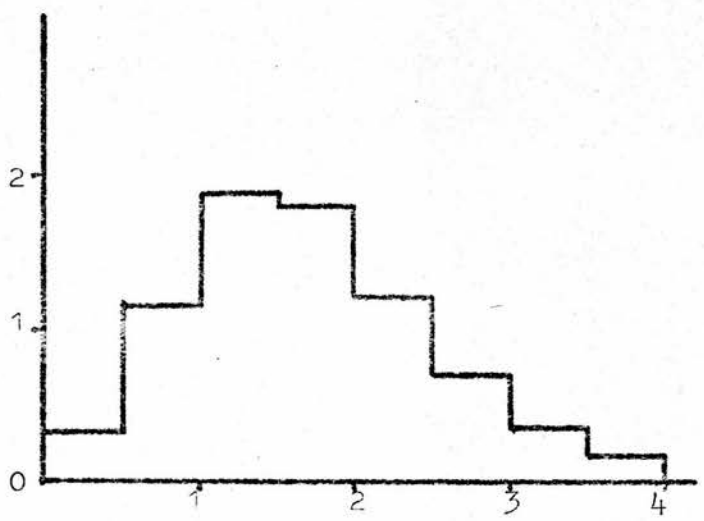
FIGURE 53.

FRACTIONAL ELUTION OF PURE STANDARDS OF ADRENALINE AND NORADRENALINE FROM 2cm AMBERLITE CG-120 COLUMNS.

a) With 1N Hydrochloric acid



b) With 1N Perchloric acid



mls →

APPENDIX III.

URINARY CATECHOLAMINE ASSAY

When radioactively labelled adrenaline or noradrenaline is injected intravenously the label compound appears in the urine as follows (See Axelrod 1965).

1 - 2% unchanged

20 - 30% ortho-methylated derivatives

60 - 70% H.M.M.A. (4-hydroxy-3-methoxymandelic acid)

It is hard to asses how relevant such studies are to the metabolism and excretion of the noradrenaline released during sympathetic activity. However, they probably serve as a reasonable index of the fate of that catecholamine which is washed out of the tissues.

On the other hand, an intravenous injection does mimic the release of catecholamines by the adrenal gland. Thus the excretion of adrenaline and its metabolite, metanephrine, has considerable potential as an index of adrenal activity in experiments on human subjects.

Only a small proportion of the injected adrenaline is excreted unchanged and thus any alteration in catecholamine metabolism could have dramatic effects on the amount of unchanged adrenaline excreted - that is, without there having been any change in adrenal discharge. As a quarter of an injection of adrenaline is excreted in the form of metanephrine, this would appear to be more representative of adrenal activity.

H.M.M.A. is only useful as an index of the total catecholamine activity as it is also formed when monoamine oxidase acts on normetanephrine and is thus the common end product for both catecholamines.

We had several reasons for developing an assay for catecholamines and their O-methylated derivatives in urine:

1. As mentioned above, the adrenaline and metaphrine levels in urine would probably reflect the activity of the adrenal gland over the period of the collection.

2. Greater concentrations are present in urine compared with plasma and this makes the assay easier.

3. It would be impracticable to study adrenal activity over an extended period of time by means of serial peripheral blood samples because of the volume of blood that would be required, - (each plasma estimation requires 10-20 mls of whole blood).

N.B. The duration of the collection periods can be greatly reduced by water loading the subjects.

4. We intended to perform some long term studies on patients using 24-hour collections.

Assay of Noradrenaline and Adrenaline

We first tried to apply our adrenal venous plasma assay directly to urine. This method is described by Häggendal (1962) who performed his plasma assay on 5-10 mls urine and does not mention any difficulties. We found, even after centrifugation and dilution, that when using larger volumes (20-40 mls) we did not obtain satisfactory fluoresence curves and our blanks were unacceptably high. This was due presumably to contaminants. We thought that the use of boric acid which selectively elutes catecholamines, should greatly reduce the contamination of the final eluate which was to be used for fluorophor formation.

Urine assays had been described by Mattock and Wilson (1966) and Sandhu and Freed (1968) in which catecholamines were eluted from weak cation exchange resins using boric acid. They reported recoveries of around 90%. However, they stressed that it is essential to dilute the urine to achieve this degree of recovery and mention their failure to assay satisfactorily some samples which had a high ionic content. Their eluting volume of boric acid was never less than half that of the initial volume of urine being subjected to assay and thus their concentration factor was only about two-fold.

We found that their technique gave good quality catecholamine fluorescence curves, but we wanted to increase the concentration of catecholamines in the final eluate and perform the assay on larger volumes of urine. Thus we applied our two-column technique, which I p273 have already described for plasma estimations, to the assay of urinary catecholamines. The results were very promising with consistent recoveries of over 80%.

ASSAY OF O-METHYLATED DERIVATIVES

Separation of Normetanephrine and Metanephrine from the Catecholamines

It is obviously advantageous to be able to perform the estimation of both pairs of amines on the same urine sample. This was done by Weil-Malherbe and Smith (1966) and Brunges, Wybenga and Johns (1964) by passing the urine through alumina which selectively adsorbs the catecholamines while retaining only insignificant amounts of the metanephrines. The metanephrines are then extracted from the sample by an ion-exchange resin. Weil-Malherbe and Smith used formic acid while Brunges' group used acetic acid for elution.

We wanted a method in which a single column could be used for the extraction and the separation. We first studied the elution pattern of pure standards from a 5 cm column of Amberlite CG-120 when using 1N hydrochloric acid and achieved some separation (Figure 54a). We then employed a resin of smaller particle size but this only slightly increased the separation. (Figure 54b). These latter columns were very slow running and we achieved even less separation when we used urine instead of pure standard solutions. (Figure 54b).

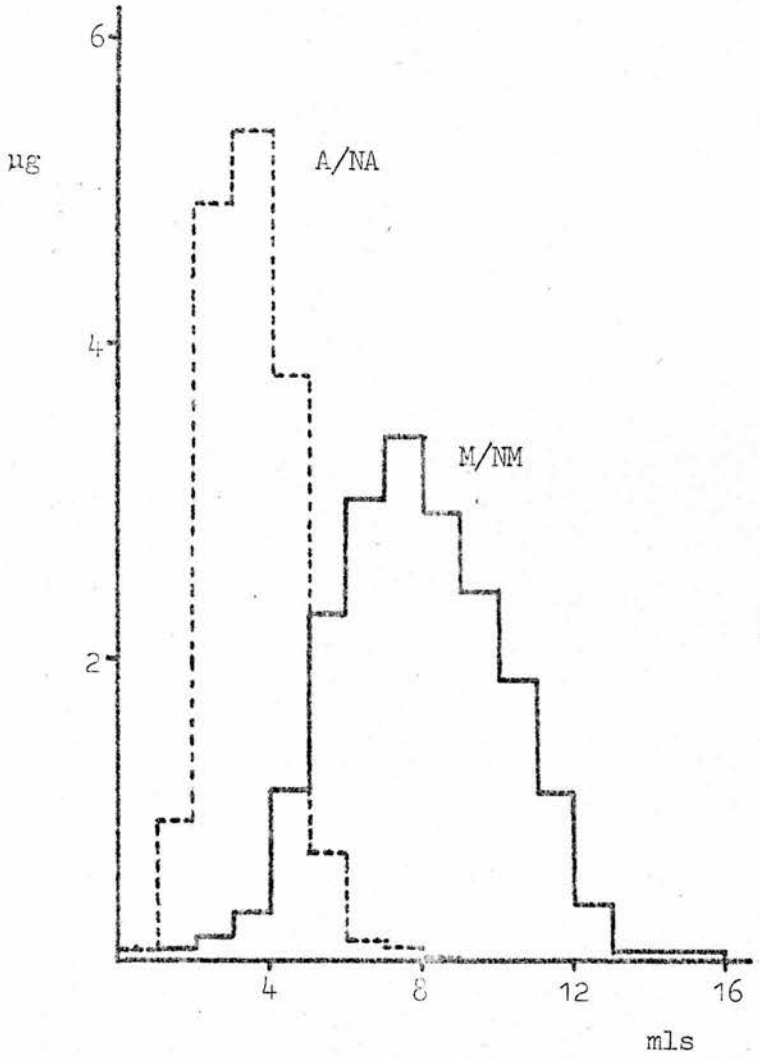
Häggendal (1962) was able to completely separate catecholamines from metanephrines by using 10-20 cm columns but such a size needs a very large elution volume and runs so slowly to be of no practical value

We decided to exploit the property of catecholamines to form complexes with boric acid which is not shared by the metanephrines.

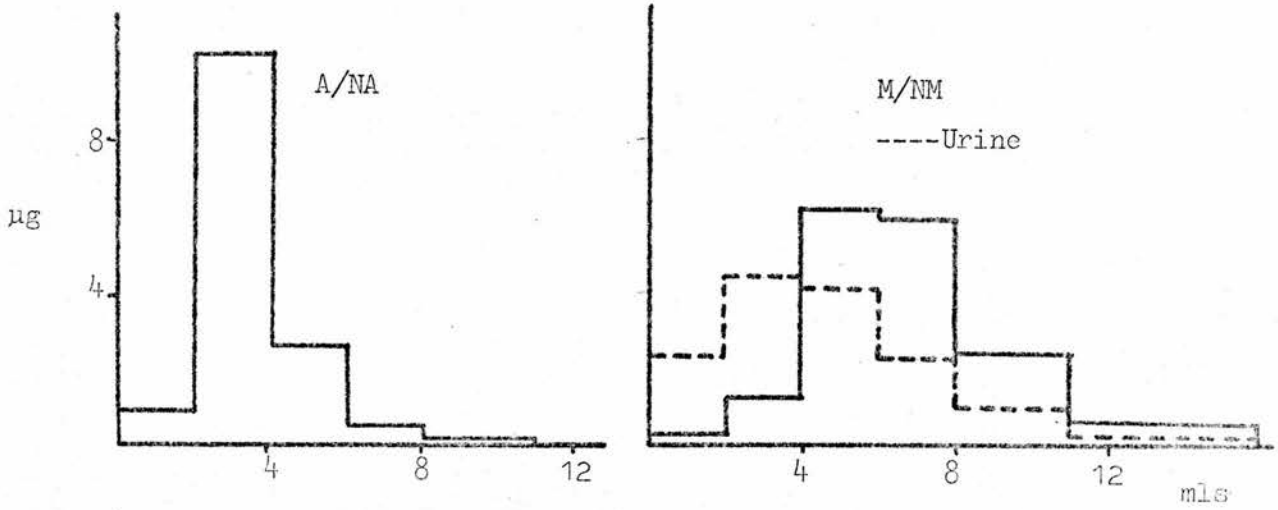
Mattock and Wilson (1966) had used a weak cation exchange resin and removed the metanephrines with dilute sulphuric acid after eluting the catecholamines with boric acid. Sandhu and Freed (1968) employed

SEPARATION OF THE CATECHOLAMINES FROM THEIR O-METHYLATED DERIVATIVES USING ION-EXCHANGE RESINS FIGURE 54.

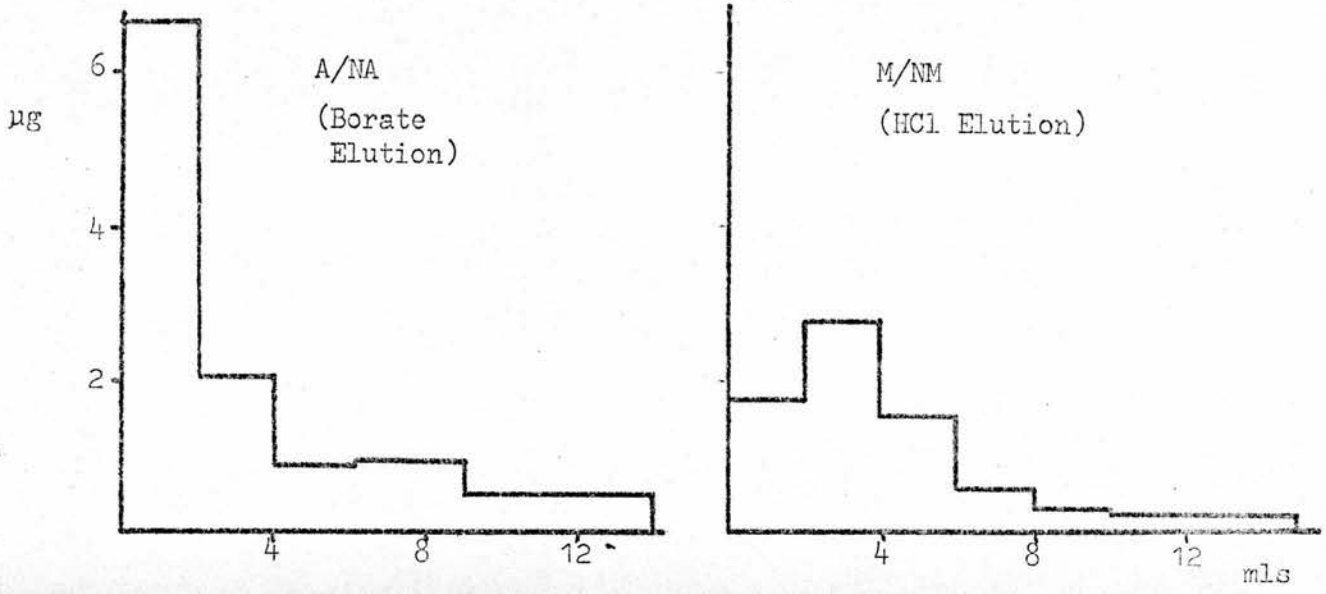
a) Elution with 1N HCl from 5cm Amberlite CG-120 columns (mesh size 100-200)



b) Elution with 1N HCl from 5cm Dowex-50 columns (mesh size 200-400)



c) Elution from 2cm Amberlite CG-120 (100-200 mesh) using a Borate solution followed by 1N HCl, acheiving complete separation



4N ammonium hydroxide to elute the metanephrines from similar columns but comment that their method is not sufficiently accurate to do more than detect, higher than normal levels, in urine.

However, the ionic content of our urine samples was very likely to vary and so we considered it preferable to use a strong rather than a weak acid cation exchange resin.

In some pilot experiments using our standard 2 cm columns of Amberlite CG 120 we first eluted the catecholamines with a borate solution (boric acid and sodium hydroxide at pH 7.5) and then eluted the metanephrines with 1N hydrochloric acid. Figure 54c shows that for complete elution large eluting volumes are required. In our recovery experiments we used 10 mls of both borate and hydrochloric acid and obtained mean recoveries of 87 $\pm$ 2% (n = 12) for the catecholamines and 70 $\pm$ 2% (n = 12) for the metanephrines. We also found that with the catecholamines the blanks were much lower than when they were eluted with hydrochloric acid.

Fluorimetric Estimation of Normetanephrine and Metanephrine

Bertler, Carlsson and Rosengren (1959) first demonstrated that the trihydroxyindole method could be applied to the O-methylated catecholamine derivatives with the production of fluorphors identical to adrenolutine and noradrenolutine, although the oxidation has different optimal conditions to those required for catecholamines. However, these differences are not sufficient for differentiation between the two pairs of amines and it is first necessary to separate metanephrine and normetanephrine from the catecholamines and other related compounds.

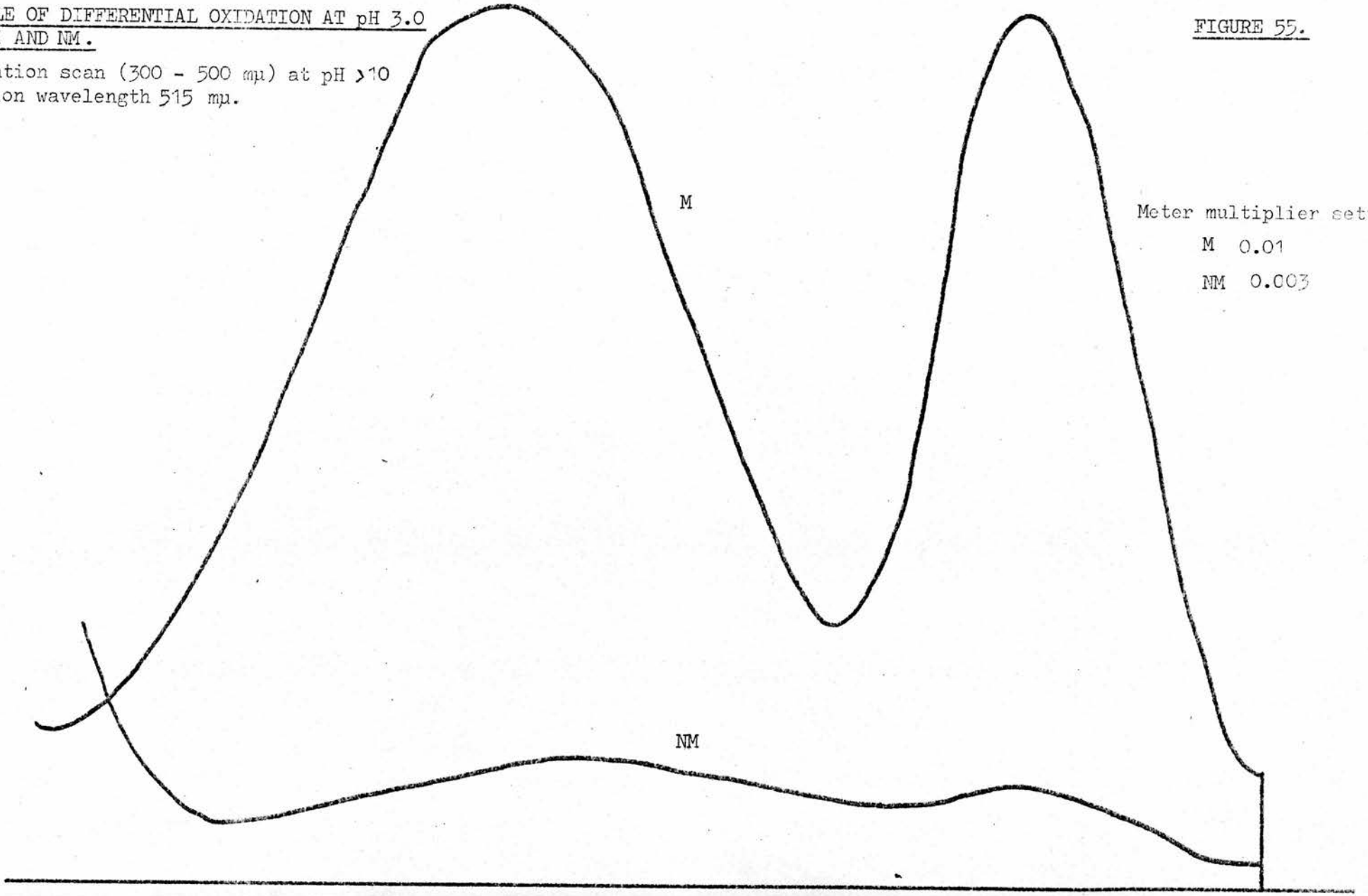
Laverty (1968) found that both metanephrine and normetanephrine are oxidised by iodine at an optimum pH of between 8.6 and 9.1. Häggendal (1962) used iodine at pH 6.5 - 7.0 and differentiated between the two compounds by reading at two separate wave lengths. Smith and Weil-Malherbe (1964) used a differential pH technique. In the latter both compounds are oxidised by iodine at pH 7-9 and metanephrine alone is oxidised by using potassium ferricyanide at pH 3 in the presence of a high concentration of zinc ions. They found that the use of potassium ferricyanide at the higher pH yielded fluorphors with less fluorescence and stability to those obtained with iodine.

Our own work confirmed this latter observation of Smith and Weil-Malherbe. We obtained stable fluorphors by oxidising metanephrine at pH 3 with potassium ferricyanide. The reaction required the presence of cupric ions in a three fold higher concentration to that used in our catecholamine assay. At this low pH no significant fluorescence was derived from normetanephrine while at a pH of around 7.5 both compounds were oxidised by potassium ferricyanide. (Figure 55)

EXAMPLE OF DIFFERENTIAL OXIDATION AT pH 3.0 OF M AND NM.

FIGURE 55.

Excitation scan (300 - 500 mμ) at pH >10
Emission wavelength 515 mμ.



Meter multiplier setting

M 0.01

NM 0.003

APPENDIX IV.

THE MEASUREMENT OF TOTAL ABDOMINAL VENOUS CAPACITANCE AND ITS INTERACTION WITH THE PORTAL VENOUS SYSTEM

Introduction

Our own experiments and those of others had suggested that there may be a difference between the baroreceptor and chemoreceptor reflexes in relation to the proportions of muscarinic to nicotinic ganglion receptors in their respective pathways (see p.229). Some of this evidence is based on studies of the relative influences of reflexes and ganglion stimulant drugs on arterial and venous tone. Thus, in order to extend the scope of our own investigations, we wanted to develop a technique for studying changes in arterial resistance and venous capacitance in the innervated preparation.

Most of the techniques available for studying resistance and capacitance, together with their theoretical background, have been reviewed by Mellander and Johansson (1968). The technique of constant flow perfusion for assessing alterations in arterial resistance was well established, and therefore, we concentrated our resources on the investigation of various methods for measuring changes in venous capacitance.

Unfortunately we did not have the resources to employ any of the methods, which we examined, in a study of the carotid reflex pathways. However, we did develop a technique for investigating changes in abdominal capacitance and, with it, we obtained evidence that there is an interaction between the portal and systemic venous capacitance systems.

This interaction may result in drugs and reflex responses having unexpected effects on venous return. Before I describe these abdominal capacitance studies, I shall briefly mention some features of the other techniques with which we have had experience.

We initially attempted to measure venous capacitance changes in the dog or cat hind limb. This site was chosen because of the small amount

of surgery required for access to the femoral artery and vein. An iso-volumetric approach was used which depended on perfusing the vascularly isolated region at an equal inflow and outflow. Thus, in this set up, the inflow perfusion pressure was the index of arterial resistance while the upstream outflow (i.e. venous) pressure was taken as the index of capacitance.

However, the Watson - Marlow (MHRE) pump does not reliably deliver two equal flows. Also it was impracticable to vascularly isolate the hind limb due to the extensive anastomosis around the head of the femur. The same problem of anastomosis would apply to the forelimb. Other workers have claimed to achieve complete vascular isolation using ligatures. However, on examination of demonstrations of this technique, we found that these permitted the passage of two fingers between the ligature and the tissue. (Physiological Society meeting of December 1973). We tried using Jubilee clips or ligating individual groups of muscle but both caused an undesirable amount of tissue trauma.

It was relatively simple to vascularly isolate a loop of small intestine but we found that, after this amount of manipulation of the gut, the preparation tended to deteriorate. Furthermore, we found that techniques based on changes in the weight of the constant flow perfused vascular region (e.g. hindlimb or small intestine loop) required an undesirable amount of surgery to seperate the tissue sufficiently from the animal to enable accurate weighing to be performed.

Hainsworth, Karim and Stoker (1974) described a technique in which changes in venous tone were determined by the pressure drop along the saphenous vein which was perfused with blood at constant flow via a small tributary in the foot. However, we found that this method required an unphysiologically high venous perfusion pressure and suffered from the theoretical disadvantage that, unless the vein was vascularly isolated,

local changes in arterial inflow along its length would also effect the pressure gradient.

The interaction between the resistance and capacitance vessels in the supply of blood to the tissues is very complex but as a broad generalisation it can be said that alterations in arterial resistance determine the regional distribution of the cardiac output while the importance of changes in venous capacitance lies with their overall effect on venous return and the cardiac output as a whole.

Thus we were interested in developing a technique which would look at overall rather than regional changes in venous capacitance. Some methods have been reported (see Mellander and Johansson, 1968) which required complicated cardiac bypass surgery but we considered that it would be economically more feasible to develope a technique to look at capacitance changes in the lower half of the body, i.e. below the diaphragm.

Hainsworth and Karim had demonstrated their abdominal capacitance technique at a Physiology Society meeting in December 1973 and we decided to use a similar technique with the following modifications:-

1) They perfused the vascularly isolated abdomen via the aorta and inferior vena cava. We found it simpler and obtained a more stable preparation by tying the aorta and inferior vena cava and perfusing in through a carotid-femoral arterial connection and out through a femoral-jugular venous connection

2) We assessed changes in venous capacitance by either an isovolumetric approach or one using our servo amplifier system which kept venous pressure constant as well as giving an electrical signal representing outflow.

3) We considered the interaction between portal and systemic venous capacitance and its effect on venous return.

Von Euler (1939) had demonstrated that a mixture of prostaglandins caused a large fall in systemic blood pressure and a major component of this fall was due to the damming back of blood within the hepatic portal system

with a consequent reduction in venous return. We thought that this type of redistribution of blood between the portal and the systemic capacitance beds could have a profound effect on the indices of capacitance obtained from techniques like that of Hainsworth and Karim (1973).

Method

We used pentobarbitone anaesthetised dogs (30 mg/Kg) in which the abdomen was vascularly isolated by ligation of the thoracic aorta, inferior vena cava and both azygos veins. The six lower intercostal arteries were also tied and the vertebral veins occluded by injecting hot wax or exothermic setting plastic cement (- expands on setting) via a lumbar puncture technique in order to completely abolish collateral flow.

The abdomen was perfused at constant flow through a femoral artery with blood taken from the carotid artery. In order to reduce the work load of the Watson Marlow (MHRE) pump we tied off the kidneys, common iliac vessels and removed the spleen.

Venous blood was removed from a femoral vein and returned to a jugular vein either:-

a) At the inflow rate giving an isovolumetric situation in which changes in venous pressure were taken as an index of changes in capacitance.

b) At constant pressure using our servo-control amplifier system (see p. 89) to control the Watson Marlow pump. The pump drive electrical output (i.e. the flow signal) was integrated and this was our index of the volume (i.e. capacitance) changes.

A cannula was passed through the splenic vein into the portal vein and enabled us to:-

a) Measure portal venous pressure.

b) Shunt blood to and from the femoral vein and thus bypass the liver. We considered using our servo-control system to help keep portal vein pressure at the same level as that in the systemic capacitance vessels (i.e. inferior vena cava - see p. 91)

Results

For each drug we performed paired tests by comparing the responses to equal doses given intra - arterially , i.e. into the femoral arterial inflow line, when the bypass was open and when it was closed. The order was alternated. Our preliminary experiments on six dogs support our view that there is an interaction between portal pooling and venous return.

1) Noradrenaline (10-200 µg) (Levophed - Winthrop) - In twelve out of sixteen paired tests we found that, when the bypass was open, there was a larger increase in venous return which was accompanied by a smaller rise in portal venous pressure.

2) Angiotensin (10 ug) - In five paired tests we observed a similar pattern of responses to those obtained with noradrenaline.

On several occasions, when the bypass was closed, both these drugs have produced an actual fall in venous return when very large rises in portal pressure occurred.

3) Prostaglandin E_2 (50-400 ug) - This resulted in a fall in venous return combined with a rise in portal venous pressure and in six out of eight paired tests we recorded a smaller fall in venous return when the bypass was open.

In one case an actual rise in venous return, associated with a smaller rise in portal venous pressure was noted.

4) Prostaglandins D_2 and $F_{2\alpha}$ (100-800 ug) - These increased venous return and portal venous pressure but tachyphylaxis prevented an investigation of the interaction phenomena.

5) Vasopressin (4-10 I.U.) - Using the capacitance method (a), this led to a rise in venous return with a rise in portal venous pressure but no change occurred using the method (b). However, with the bypass open, method (a) showed the larger rise in venous return and method (b) showed a rise in venous return combined with a fall in portal venous pressure.

Discussion

Our results indicate that there is an interaction between the systemic (i.e. parietal) and splanchnic capacitance beds. Consequently we felt that the phenomenon of hepatic portal pooling should be considered when interpreting the action of vasoactive drugs on venous return.

We suggest that the following mechanisms may be involved in hepatic portal pooling with an accompanying reduction in venous return to the heart.

A) That associated with a fall in portal venous pressure:-

-Portal and splanchnic vein dilatation.

B) Those associated with a rise in portal venous pressure:-

i) Constriction of hepatic and portal venule sphincters or dilatation of hepatic arterioles resulting in an apparent increase in hepatic resistance.

ii) Dilatation of splanchnic arterioles resulting in increased flow through the splanchnic bed at the expense of parietal blood flow.

We consider that our technique is potentially useful for investigating the effects of drugs and reflexes on arterial resistance and venous capacitance. Not only can it assess the interaction between portal and systemic capacitance but also,by using appropriately placed electromagnetic flow probes,regional changes in arterial resistance can be studied.

It also has the advantage that the vascular territory under study (i.e. the whole of the animal below the diaphragm) is subjected to relatively little surgery.

APPENDIX V.

SOME QUESTIONS

I would like to finish by asking a number of questions. If one assumes that the selective neural and humoral mechanisms are found in the same species, then, how is the evidence for the selective innervation of adrenaline and noradrenaline cells with different proportions of muscarinic and nicotinic receptors reconciled with the phenomenom of the induction of PNMT by corticosteroids? What mixture of catecholamines would chemoreceptor stimulation release in a hypophysectomised cat?

The internal circulation of the adrenal gland is complex. Does it influence catecholamine release through local changes in the distribution of gland blood flow? The ungulates are particularly interesting as the arteriae medullae supply areas containing solely noradrenaline. (see p. 68)

What is the short term fate of circulating adrenaline? Is some taken up by the adrenergic neurones and released with the noradrenaline? If this is the case, then the adrenaline proportion of the adrenergic neurotransmitter may rise in conditions of chronic adrenal activation.

What is the role of adrenal noradrenaline? Vane (1969) claims that adrenal catecholamines play little part in the haemodynamic responses to reflexes such as those from the baroreceptors and this view is supported by the work of Celander (1954). One possible role is that of "topping up" peripheral adrenergic neurone stores under conditions of prolonged stress in which synthesis and reuptake may not be able to keep pace with the loss to extraneuronal uptake sites.

APPENDIX VI. TABLES OF RESULTS

INDEX

UNITS

Carotid Sinus Pressure (CSP) - mmHg

Systemic Blood Pressure (SBP) - mmHg

Arterial Blood Gas Tensions - torr

Catecholamines- Whole Animals - ng/kg.min

Isolated Glands - ng/min

Adrenal Gland Blood Flow - mls/min

DR1 32kg (M)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	152	186	0.84	4.46	5.29	15.8	5.6
2	1½	BARO	103	32	7.42	95	293	1.67	11.4	13.1	12.7	6.4
3	10	C	44	27	7.44	156	173	0.99	4.18	5.16	19.1	5.8
4	11½	CHEMO	28	37	7.40	156	206	0.99	4.18	5.16	19.1	4.6
5	20	C	-	-	-	143	93	-	-	-	-	4.6
6	21½	BARO	126	31	7.38	78	160	-	-	-	-	4.6
7	30	C	47	30	7.42	143	146	0.62	4.20	4.82	12.9	4.2
8	31½	CHEMO	27	30	7.37	143	213	1.22	5.68	6.90	17.7	4.6
	34	GUANETHIDINE (2mg/kg)										
9	40	C	-	-	-	134	93	1.18	4.03	5.21	22.6	3.4
10	41½	BARO	154	36	7.40	73	93	2.76	9.38	12.1	22.8	3.4
11	50	C	47	34	7.40	130	100	0.60	3.83	4.43	13.5	4.2
12	51½	CHEMO	27	43	7.35	130	106	0.60	3.83	4.43	13.5	4.6
13	60	C	-	-	-	139	100	0.28	1.78	2.06	13.5	4.8
14	61½	BARO	140	35	7.41	73	126	1.14	5.76	6.90	16.5	4.8
15	70	C	47	34	7.42	139	93	1.46	5.12	6.58	22.1	4.6
16	71½	CHEMO	24	40	7.38	139	120	2.12	7.60	9.73	21.8	5.0

DR2 32kg (M)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	-	-	1.99	4.99	6.98	28.5	5.9
2	1½	BARO	122	37	7.37	-	-	1.99	6.38	8.38	23.8	5.9
3	10	C	58	42	7.37	-	-	2.59	9.75	12.4	21.0	5.4
4	11½	CHEMO	32	58	7.34	-	-	3.64	11.1	14.7	24.8	6.0
5	20	C	-	-	-	-	-	2.76	10.9	13.6	20.3	4.7
6	21½	BARO	83	41	7.35	-	-	5.40	17.4	22.8	23.6	5.5
7	30	C	54	43	7.34	-	-	5.93	18.5	24.5	24.3	5.0
8	31½	CHEMO	33	58	7.28	-	-	6.06	19.9	25.9	23.4	6.4
	33	GUANETHIDINE (2mg/kg)										
9	40	C	-	-	-	-	-	3.47	13.8	17.3	20.0	4.0
10	41½	BARO	155	42	7.32	-	-	9.60	24.7	34.3	28.0	4.2
11	50	C	55	42	7.35	-	-	5.61	23.5	29.1	19.3	3.7
12	51½	CHEMO	27	62	7.26	-	-	9.98	34.3	44.3	22.5	4.0

DR3 23kg (M)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	136	140	2.21	12.6	14.9	14.9	6.4
2	1½	BARO	90	33	7.40	75	250	-	-	-	-	6.3
3	10	C	91	42	7.39	136	140	0.81	7.45	8.26	9.7	6.4
4	11½	CHEMO	41	43	7.37	136	140	1.37	9.69	11.1	12.4	6.4
5	20	C	-	-	-	142	118	1.79	12.2	14.0	12.8	6.9
6	21½	BARO	89	34	7.41	75	222	5.07	39.6	44.6	11.4	8.4
7	30	C	81	44	7.38	142	118	2.21	15.3	17.5	12.6	6.0
8	31½	CHEMO	34	44	7.40	142	118	2.47	15.4	17.9	13.9	6.4
	33	GUANETHIDINE (2mg/kg)										
9	40	C	-	-	-	139	72	0.95	4.89	5.84	16.3	4.3
10	41½	BARO	78	39	7.39	75	72	9.24	25.0	34.2	27.0	4.3
11	50	C	87	36	7.39	139	72	0.95	4.89	5.84	16.3	5.5
12	51½	CHEMO	35	41	7.38	139	72	2.86	8.02	10.9	26.3	5.5
13	60	C	-	-	-	136	72	1.28	6.68	7.96	16.1	5.9
14	61½	BARO	82	39	7.39	75	72	7.27	27.1	34.3	21.1	7.3
15	70	C	92	34	7.43	136	72	2.95	9.21	12.2	24.3	5.2
16	71½	CHEMO	33	41	7.39	136	72	2.95	15.5	18.5	16.0	4.5
17	80	C	-	-	-	136	77	0.78	6.02	7.00	11.4	6.7
18	81½	BARO	96	35	7.43	75	104	5.22	27.1	32.3	16.1	7.9
19	90	C	90	36	7.43	136	77	1.28	7.10	8.38	15.3	5.8
20	91½	CHEMO	36	40	7.42	136	77	1.64	10.1	11.7	16.4	5.0

DR4 28kg (M)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	147	44	0.47	1.69	2.06	22.8	2.2
2	1½	BARO	89	60	7.21	90	146	2.57	6.68	9.25	27.8	6.0
3	10	C	114	50	7.23	147	53	2.69	7.93	10.6	25.3	2.9
4	11½	CHEMO	42	70	7.18	147	111	3.67	11.7	15.4	23.8	4.4
5	20	C	-	-	-	147	48	0.71	5.14	5.85	12.1	2.6
6	21½	BARO	84	52	7.26	90	120	2.40	12.1	14.47	16.6	5.0
7	40	C	91	52	7.31	147	44	1.05	13.1	14.1	7.5	2.0
8	41½	CHEMO	22	68	7.10	147	124	4.50	19.6	24.1	18.7	3.4
9	50	C	-	-	-	147	35	1.71	13.0	14.7	11.6	2.5
10	51½	BARO	94	48	7.30	90	97	2.50	15.7	18.2	13.7	4.4
11	60	C	95	52	7.25	150	31	0.54	3.84	4.38	12.3	2.0
12	61½	CHEMO	41	76	7.05	150	88	3.55	27.0	30.5	11.6	4.7
13	70	C	-	-	-	-	-	1.08	6.90	7.98	13.5	2.1
14	71½	BARO	95	54	7.24	-	-	6.95	29.5	36.5	19.1	3.9
15	80	C	89	48	7.25	-	-	0.76	2.55	3.31	23.0	1.5
16	81½	CHEMO	2	104	6.98	-	-	6.27	26.0	32.2	19.4	1.8

DR5 13.5 (F)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	155	56	1.12	2.29	3.40	32.8	7.3
2	1½	BARO	100	43	7.34	75	122	1.32	9.40	10.7	12.3	10.5
3	10	C	-	-	-	155	44	1.22	3.45	4.67	26.1	6.0
4	11½	BARO	-	-	-	80	97	1.63	11.9	13.6	12.0	8.0
5	20	C	-	-	-	155	47	-	-	-	-	4.5
6	21½	BARO	110	24	7.34	80	100	1.47	9.90	11.4	12.9	7.3
7	31½	BARO	119	24	7.46	-	-	-	-	-	-	7.4
8	35	C	-	-	-	-	-	0.36	5.18	5.54	6.4	3.6
9	40	C	119	23	7.41	160	31	0.56	0.97	1.52	36.7	3.4
10	41½	BARO	111	23	7.37	80	103	1.17	4.37	5.54	21.1	6.6
11	50	C	-	-	-	150	44	1.17	1.27	2.44	47.9	3.6
12	51½	BARO	105	38	7.32	80	141	1.27	7.26	8.53	14.9	6.8

DN1 11.5kg (F)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	425	44	7.37	-	-	7.63	30.7	38.3	19.9	-
2	2	STIM	-	-	-	-	-	131.5	451.1	582.6	22.6	-
3	7	C	-	-	-	-	-	8.41	30.3	38.8	21.7	6.4
4	9	STIM	-	-	-	-	-	70.1	295.7	365.7	19.2	7.0
5	16	C	-	-	-	-	-	9.06	35.3	44.4	20.4	6.7
6	18	STIM	-	-	-	-	-	53.2	197.9	251.1	21.2	8.0
7	32	C	410	42	7.25	-	-	6.44	23.8	30.2	21.3	7.3
8	33	STIM	-	-	-	-	-	57.7	184.2	241.8	23.8	7.7
9	43	C	425	46	7.31	-	-	7.27	20.2	27.4	26.5	6.0
10	45	STIM	-	-	-	-	-	55.7	174.4	230.1	24.2	7.0
11	53	C	-	-	-	-	-	10.3	25.7	36.0	28.7	6.0
12	55	STIM	-	-	-	-	-	77.2	191.3	268.5	28.8	7.0
		ATROPINE (20µg/kg)										
13	66	C	427	43	7.30	-	-	10.1	22.2	32.1	31.4	4.4
14	68	STIM	-	-	-	-	-	46.5	149.9	196.4	23.7	3.5
		HEXAMETHONIUM (2mg/kg)										
15	73	C	-	-	-	-	-	0.24	0.84	1.07	22.2	-
16	75	STIM	-	-	-	-	-	0.24	0.84	1.07	22.2	0.8

DN2 12kg (F)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	420	38	7.31	-	-	3.77	20.1	23.9	15.8	6.7
2	1⅓	STIM	-	-	-	-	-	4.74	29.3	34.1	13.9	6.6
3	5	C	-	-	-	-	-	1.89	8.00	9.89	19.1	7.0
4	6⅓	STIM	-	-	-	-	-	9.31	44.5	53.8	17.3	7.0
5	10	C	420	40	7.38	-	-	1.20	6.63	7.83	15.3	6.5
6	14	C	-	-	-	-	-	1.26	6.11	7.37	17.1	7.5
7	15⅓	STIM	-	-	-	-	-	19.5	104.1	123.7	15.8	9.0
8	20	C	-	-	-	-	-	1.54	6.29	7.83	19.7	6.5
9	21⅓	STIM	-	-	-	-	-	22.3	91.1	113.4	19.7	6.5
10	30	C	415	39	7.37	-	-	0.40	2.46	2.86	14.0	5.0
11	31⅓	STIM	-	-	-	-	-	2.34	6.91	9.26	25.3	5.5
12	40	C	420	38	7.37	-	-	1.20	3.20	4.40	27.3	4.9
13	41⅓	STIM	-	-	-	-	-	2.63	4.74	7.37	35.7	5.5
	42½	HEXAMETHONIUM (2mg/kg)										
14	46½	C	-	-	-	-	-	0.23	2.69	2.91	7.8	2.0
15	47¾	STIM	-	-	-	-	-	1.31	5.14	6.46	20.4	2.7
	50	ATROPINE (20µg/kg)										
16	54	C	-	-	-	-	-	0.46	5.31	5.77	7.9	1.0
17	55⅓	STIM	-	-	-	-	-	0.46	5.31	5.77	7.9	1.0

DN3 12kg (F)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	390	44	7.30	-	-	0.80	7.94	8.74	9.2	4.0
2	1½	STIM	-	-	-	-	-	0.86	15.4	16.3	5.3	3.8
3	5	C	-	-	-	-	-	3.37	17.7	21.1	16.0	3.9
4	6½	STIM	-	-	-	-	-	2.34	37.9	40.3	5.8	5.6
5	10	C	405	45	7.36	-	-	1.77	24.4	26.2	6.8	4.4
6	11½	STIM	-	-	-	-	-	2.23	26.0	28.2	7.9	4.5
7	15	C	-	-	-	-	-	2.29	25.3	27.5	8.3	4.4
8	16½	STIM	-	-	-	-	-	2.06	54.6	56.6	3.6	4.4
	17	ACTH (iv) (1 IU/kg)										
9	22	C	-	-	-	-	-	4.34	26.5	30.8	14.1	5.0
10	23½	STIM	-	-	-	-	-	1.31	23.9	25.3	5.2	6.6
11	27	C	385	43	7.36	-	-	4.23	47.8	52.0	8.1	4.9
12	28½	STIM	-	-	-	-	-	1.83	96.7	98.6	1.9	4.9
13	32	C	-	-	-	-	-	31.8	145.2	177.0	18.0	4.2
14	33½	STIM	-	-	-	-	-	30.7	139.6	170.3	18.0	4.0
15	37	C	-	-	-	-	-	33.9	156.4	190.3	17.8	3.7
16	44	C	380	45	7.34	-	-	12.4	96.7	109.1	11.4	3.1
17	45½	STIM	-	-	-	-	-	21.1	107.3	128.3	16.4	3.5
18	49	C	-	-	-	-	-	38.2	160.7	198.9	19.2	3.4
19	50½	STIM	-	-	-	-	-	51.1	193.0	244.2	20.9	3.2
	55	HEXAMETHONIUM (2mg/kg)										
20	60	C	-	-	-	-	-	0.66	2.66	3.32	19.8	1.2
21	62½	STIM	-	-	-	-	-	2.29	10.1	12.4	18.4	1.0

	DN3	12kg (F)										
22	67	C	-	-	-	-	-	0.80	3.57	4.37	18.3	1.3
	70	ATROPINE (20µg/kg)										
23	74	C	-	-	-	-	-	1.20	4.97	6.17	19.4	0.8
24	76½	STIM	-	-	-	-	-	1.03	9.37	10.4	10.0	0.7

DA1 23.5kg (M)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	143	106	1.43	6.32	7.75	18.5	4.5
2	1½	BARO	395	53	7.28	85	184	3.31	9.11	12.4	26.7	4.9
3	5	C	-	-	-	143	91	1.51	6.63	8.14	18.6	4.1
4	6½	BARO	420	54	7.24	106	138	2.81	10.7	13.5	20.8	4.5
5	10	C	-	-	-	140	84	1.39	6.86	8.24	16.8	4.2
6	11½	BARO	410	51	7.24	60	153	3.40	11.5	14.9	22.8	4.6
7	20	C	-	-	-	137	81	1.68	7.25	8.92	18.8	4.0
8	21½	BARO	430	58	7.23	72	122	2.22	9.94	12.2	18.2	4.9
9	30	C	-	-	-	137	66	1.31	7.44	8.76	15.0	4.0
10	31½	BARO	390	44	7.32	72	128	-	-	-	-	4.8
	33	ACTH (25I.U.) (33-48mins)										
11	40	C	-	-	-	143	66	4.78	11.7	16.5	29.0	4.4
12	41½	BARO	365	41	7.30	72	119	0.22	20.8	21.1	1.1	5.1
13	45	C	-	-	-	143	63	1.33	13.6	14.9	8.4	4.2
14	46½	BARO	400	45	7.30	75	122	8.00	19.5	27.5	29.1	4.9
15	50	C	-	-	-	140	56	4.15	11.5	15.7	26.5	4.2
16	51½	BARO	375	40	7.31	75	116	8.51	21.3	29.8	28.5	4.6
17	65	C	-	-	-	137	50	5.50	21.9	27.4	20.1	4.4
18	66½	BARO	430	42	7.28	72	91	9.89	26.4	36.3	27.2	4.8
19	80	C	-	-	-	137	47	7.23	25.0	32.2	22.5	4.0
20	81½	BARO	455	45	7.29	69	75	5.21	45.2	50.4	10.3	4.5

DA1 23.5kg (M)

21	85	C	-	-	-	131	38	3.50	14.5	18.0	19.4	3.8
22	86½	BARO	445	47	7.27	109	50	8.63	21.8	30.4	28.4	4.0
23	90	C	-	-	-	131	38	1.80	23.8	25.6	7.1	4.0
24	91½	BARO	425	40	7.29	57	66	5.94	39.0	45.0	13.2	4.6

DA2 31kg (M)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	163	93	0.86	4.82	5.68	15.2	7.8
2	1½	BARO	220	45	7.31	94	193	8.51	24.0	32.5	26.2	11.3
3	10	C	-	-	-	163	96	2.41	6.41	8.82	27.3	5.2
4	11½	BARO	422	40	7.35	89	203	1.84	5.22	7.05	26.0	6.8
5	15	C	-	-	-	168	90	1.33	6.63	7.96	16.7	5.4
6	16½	BARO	385	42	7.35	89	203	7.87	27.0	34.9	22.6	11.5
	18	ACTH (iv) (25 IU.20 min infusion)										
7	25	C	-	-	-	168	93	2.06	8.60	10.7	19.3	5.9
8	26½	BARO	390	44	7.31	89	181	6.88	49.9	56.8	12.1	11.3
9	30	C	-	-	-	168	90	2.08	14.5	16.6	12.5	5.6
10	31½	BARO	395	43	7.34	89	148	10.0	48.3	58.3	17.2	7.1
11	40	C	-	-	-	168	72	1.77	15.1	16.8	10.5	4.4
12	41½	BARO	360	42	7.28	89	133	8.65	43.3	51.9	16.6	10.6
13	45	C	-	-	-	168	69	3.74	17.8	21.6	17.3	5.6
14	46½	BARO	365	44	7.29	89	112	19.7	69.1	88.9	22.2	8.4
15	60	C	-	-	-	168	63	7.47	26.2	33.7	22.2	3.2
16	61½	BARO	360	39	7.31	89	127	22.7	79.5	102.2	22.2	5.4
17	75	C	-	-	-	168	78	7.74	19.5	27.3	28.4	5.0
18	76½	BARO	365	38	7.31	89	139	13.8	58.7	72.5	19.0	8.2
19	90	C	-	-	-	168	78	3.47	15.3	18.8	18.5	5.4
20	91½	BARO	365	38	7.31	89	139	14.0	36.4	58.4	27.8	8.2

DA3 6.5kg (M)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	124	57	1.66	16.2	17.9	9.3	4.0
2	2¼	BARO	365	46	7.26	85	152	20.6	88.8	109.5	18.9	6.5
3	10	C	365	44	7.33	-	-	2.50	17.4	19.9	12.6	3.2
4	12¼	C	-	-	-	-	-	1.86	16.0	17.8	10.4	2.5
	15	ACTH (5I.U.)										
5	22	C	-	-	-	-	-	8.47	39.2	47.7	17.8	4.0
6	25	C	-	-	-	-	-	6.40	34.6	41.0	15.6	3.3
7	32	C	345	44	7.33	118	38	2.00	21.1	23.1	8.5	3.4
8	34¼	BARO	-	-	-	79	100	13.0	115.6	128.6	10.1	8.0
9	42	C	-	-	-	115	29	1.63	12.7	14.3	11.4	2.5
10	44¼	BARO	335	42	7.35	76	52	14.4	83.7	98.1	14.7	5.0
11	52	C	350	41	7.31	-	-	1.59	8.20	9.80	16.3	2.5
12	54	C	-	-	-	-	-	0.95	13.5	14.5	6.6	2.0

DC1 28kg (F)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	142	112	1.81	9.65	11.5	15.8	10.8
2	1½	BARO	365	37	7.38	76	278	2.11	14.8	16.9	12.4	16.2
3	15	C	-	-	-	142	103	2.86	9.96	12.8	22.3	9.8
4	16½	BARO	70	39	7·39	80	246	3.40	19.4	22.8	14.9	16.9
		PROLONGED CHEMO STIMULATION (20-40mins) & ADRENAL GLAND DENERVATED (18mins)										
5	25	C	-	-	-	142	93	3.87	10.4	14.3	27.1	9.9
6	26½	BARO	32	46	7.34	80	181	2.33	7.79	10.1	23.0	12.9
7	35	C	-	-	-	142	84	4.85	12.8	17.7	27.5	8.8
8	36½	BARO	32	45	7.35	80	165	4.53	13.3	17.8	25.4	12.6
9	45	C	-	-	-	142	90	2.72	20.8	23.5	11.6	7.3
10	46½	BARO	91	41	7.34	80	203	3.07	17.3	20.4	15.1	17.1
11	55	C	-	-	-	142	78	8.86	28.5	37.4	23.7	7.3
12	56½	BARO	91	41	7.34	80	225	3.67	24.7	28.4	12.9	18.4
13	65	C	89	45	7.32	142	75	11.9	28.8	40.7	29.2	6.4
14	66½	CHEMO	33	60	7.30	142	134	3.76	22.2	26.0	14.5	11.5
15	75	C	73	46	7.33	147	62	4.11	25.5	29.6	13.9	4.0
16	76½	CHEMO	27	80	7.13	147	168	4.21	21.3	25.5	16.5	10.3
17	85	C	-	-	-	142	87	4.58	24.7	29.3	15.6	6.5
18	86½	BARO	92	49	7.31	80	200	4.73	26.5	31.2	15.1	14.4
19	90	C	-	-	-	-	-	9.23	34.1	43.3	21.3	-

DC2 13.5kg (F)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	100	30	7.34	-	-	2.39	21.3	23.7	10.1	1.0
2	5	C	-	-	-	-	-	3.71	23.3	27.0	13.7	1.0
3	6½	BARO	-	-	-	-	-	21.9	140.8	162.8	13.5	1.4
4	9	C	-	-	-	-	-	-	-	-	-	-
		PROLONGED CHEMO STIMULATION (10-20mins)										
	15		28	28	7.35							
5	25	C	110	24	7.36	-	-	27.9	142.3	170.2	16.4	1.2
6	30	C	-	-	-	-	-	31.5	141.9	173.5	18.2	2.6
7	35	C	119	24	7.36	-	-	3.60	139.5	143.1	2.5	2.6
8	40	C	-	-	-	-	-	19.2	105.3	124.4	15.4	5.2
	41	CYCLOHEXIMIDE (700mg)										
9	45	C	-	-	-	-	-	7.21	38.1	45.4	15.9	2.4
10	50	C	119	23	7.41	-	-	4.72	33.3	38.0	12.4	1.6
11	51½	BARO	119	23	7.41	-	-	42.7	156.2	198.8	21.5	5.2
12	54	C	-	-	-	-	-	4.52	36.5	41.0	11.0	2.0
		PROLONGED CHEMO STIMULATION (55-65mins)										
	60		26	31	7.30							
13	70	C	-	-	-	-	-	1.57	19.7	21.3	7.4	0.4
14	75	C	-	-	-	-	-	3.66	28.8	32.5	11.3	0.8
15	80	C	105	38	7.32	-	-	17.5	101.1	118.6	14.7	1.2
16	81½	BARO	105	38	7.32	-	-	27.8	192.7	220.5	12.6	4.8

DC3 10kg (M)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng3kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	-	-	-	-	11.9	29.5	0.6
2	1	C	-	-	-	150	56	-	-	14.8	26.3	0.8
3	$2\frac{1}{2}$	BARO	81	35	7.31	82	137	-	-	68.9	36.1	1.8
4	9	C	-	-	-	-	-	-	-	7.80	22.1	0.6
		PROLONGED CHEMO STIMULATION (10-20mins)										
5	15	CHEMO	35	52	7.24	154	63	-	-	10.3	26.6	0.6
6	17	CHEMO	35	52	7.24	-	-	-	-	10.6	27.4	0.7
7	26	C	-	-	-	-	-	-	-	24.9	26.6	0.6
8	28	C	-	-	-	150	63	-	-	15.6	28.4	0.6
9	$29\frac{1}{2}$	BARO	85	38	7.32	82	159	-	-	93.4	38.5	1.2
10	40	C	-	-	-	-	-	-	-	10.8	25.8	0.6

DC4 12.5kg (F)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	-	-	12.5	47.5	60.0	20.8	8.0
2	1	C	-	-	-	110	70	9.33	41.0	50.3	18.5	8.0
3	2½	BARO	74	38	7.30	67	122	18.3	94.1	112.4	16.3	7.0
4	9	C	-	-	-	110	51	5.65	38.7	44.4	12.7	7.0
		PROLONGED CHEMO STIMULATION (10-20mins)										
5	15	CHEMO	47	44	7.22	110	81	7.57	34.8	42.4	17.9	7.0
6	19	CHEMO	43	44	7.22	110	81	5.87	28.7	34.6	17.0	4.5
7	24	C	-	-	-	-	-	7.35	43.9	51.3	14.3	2.5
8	30	C	-	-	-	-	-	5.38	34.1	39.4	13.6	5.3
9	35	C	-	-	-	-	-	4.50	31.4	35.9	12.5	2.8
10	40	C	-	-	-	110	40	3.57	33.1	36.7	9.7	2.0
11	41½	BARO	86	31	7.31	67	74	23.4	140.0	164.0	14.2	7.0
12	45	C	-	-	-	-	-	5.81	46.6	52.4	11.1	4.4
	53	CYCLOHEXIMIDE (50mg/kg)										
13	55	C	-	-	-	-	-	3.24	13.7	16.9	19.2	4.5
14	60	C	-	-	-	110	59	2.69	26.6	29.3	9.2	4.0
15	61½	BARO	82	32	7.27	67	88	8.67	38.0	46.6	18.6	4.0
16	65	C	-	-	-	110	55	4.22	14.9	19.1	22.1	1.9
		PROLONGED CHEMO STIMULATION (70-80mins)										
17	74	CHEMO	37	44	7.16	110	33	8.67	34.9	43.6	19.9	2.4
18	79	CHEMO	-	-	-	110	33	4.50	24.5	29.0	15.5	1.3
19	86	C	-	-	-	-	-	5.81	27.2	33.0	17.6	1.6
20	90	C	-	-	-	110	44	4.55	28.3	32.9	13.9	1.5
21	91½	BARO	96	41	7.24	67	70	23.0	93.6	116.6	19.7	4.0

DC5 14kg (F)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	-	-	1.62	20.7	22.3	7.2	3.0
2	1	C	-	-	-	128	57	2.55	22.9	25.5	10.0	2.8
3	2½	BARO	396	46	7.29	75	157	10.4	41.8	52.3	20.0	5.4
4	8	C	-	-	-	135	64	2.50	14.5	17.0	14.7	3.3
		PROLONGED CHEMO STIMULATION (10-20mins)										
5	16	CHEMO	55	74	7.26	100	107	8.96	50.5	59.5	15.1	5.0
6	19	CHEMO	43	88	7.30	100	107	7.30	57.5	64.8	11.3	4.2
7	25	C	-	-	-	-	-	-	-	-	-	3.9
8	28	C	-	-	-	-	-	-	-	-	-	-
9	32	C	-	-	-	128	50	2.60	33.1	35.7	7.3	4.0
10	33½	BARO	109	51	7.23	75	139	9.99	72.8	82.8	12.1	6.5
11	36	C	-	-	-	-	-	3.82	22.2	26.1	14.7	4.0
12	40	C	116	62	7.27	-	-	2.60	27.9	30.5	8.5	2.5
	41	CYCLOHEXIMIDE (50mg/kg)										
13	45	C	-	-	-	-	-	0.78	22.4	23.2	3.4	4.5
14	55	C	-	-	-	-	-	2.06	12.0	14.1	14.6	3.0
15	57	C	-	-	-	132	57	0.88	11.6	12.4	7.1	2.6
16	58½	BARO	126	52	7.20	71	139	8.77	46.6	55.3	15.8	6.7
17	63	C	-	-	-	135	64	0.54	7.35	7.88	6.8	2.8
		PROLONGED CHEMO STIMULATION (70-80mins)										
18	72	CHEMO	31	76	7.20	100	75	4.65	27.9	32.5	14.3	3.5
19	76	CHEMO	31	76	7.20	100	75	3.28	30.0	33.3	9.9	3.5

	DC5	14kg (F)										
20	82	C	130	53	7.15	-	-	0.59	5.53	6.12	9.6	2.0
21	85	C	-	-	-	128	50	0.00	7.74	7.74	0.0	1.5
22	86½	BARO	127	56	7.15	71	142	5.19	25.9	31.0	16.7	4.4
23	90	C	-	-	-	-	-	0.74	3.48	4.21	17.4	1.0
	94	ACTH (1I.U./kg)										
24	100	C	-	-	-	-	-	2.01	8.03	10.0	20.0	2.0
25	105	C	-	-	-	132	39	3.18	7.69	10.9	29.3	1.8
26	106½	BARO	123	62	7.12	71	135	1.76	34.4	36.1	4.9	4.3

DC6 11.8kg

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD P O_2	P CO_2	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	-	-	0.99	4.83	5.82	17.0	1.6
2	1	C	-	-	-	125	85	1.28	2.79	4.07	31.4	1.7
3	2½	BARO	-	-	-	78	118	4.59	14.4	18.9	24.2	6.1
4	6	C	110	34	7.28	125	74	1.08	2.88	3.98	27.0	2.3
		PROLONGED CHEMO STIMULATION (10-20mins)										
5	13	CHEMO	44	47	7.27	125	100	2.38	7.03	9.41	25.3	3.4
6	19	CHEMO	40	49	7.28	125	100	0.58	9.88	10.5	5.6	1.5
7	23	C	-	-	-	-	-	1.05	7.61	8.66	12.1	1.7
8	27	C	-	-	-	125	48	1.16	6.57	7.73	15.0	2.0
9	28½	BARO	110	43	7.23	78	103	4.18	10.6	14.8	28.2	5.0
10	35	C	-	-	-	-	-	0.17	6.16	6.33	2.8	1.9
11	41	C	96	43	7.32	-	-	1.28	3.02	4.30	29.7	1.0
12	45	C	-	-	-	-	-	-	-	-	-	1.3
	50	CYCLOHEXIMIDE (50mg/kg)										
13	55	C	-	-	-	-	-	0.76	1.86	2.62	28.9	1.6
14	60	C	-	-	-	125	62	0.06	1.63	1.69	3.4	1.2
15	61½	BARO	70	52	7.18	78	100	1.28	4.01	5.29	24.2	2.2
16	67	C	-	-	-	125	44	0.06	1.34	1.40	4.2	0.5
		PROLONGED CHEMO STIMULATION (70-80mins)										
17	73	CHEMO	37	58	7.14	125	48	0.23	1.51	1.74	13.3	0.8
18	75	CHEMO	28	62	7.18	125	48	0.23	0.58	0.81	28.6	0.5

DC6 11.8kg

19	85	C	-	-	-	-	-	0.06	0.12	0.17	33.3	0.3
20	90	C	-	-	-	125	51	0.00	0.12	0.12	0.0	0.3
21	95	BARO	125	45	7.31	78	77	0.12	1.00	1.10	10.5	0.8
22	97	C	-	-	-	-	-	0.00	0.17	0.17	0.0	0.5
	98	ACTH (iv) (0.9I.U./kg)										
23	102	C	-	-	-	-	-	0.00	0.81	0.81	0.0	0.5
24	107	C	148	40	7.38	-	-	0.00	0.81	0.81	0.0	0.6

DC7 12kg (M)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	-	-	2.97	2.80	5.77	51.5	1.5
2	1	C	-	-	-	120	67	0.63	5.54	6.17	10.2	1.4
3	$2\frac{1}{2}$	BARO	135	37	7.25	75	142	10.1	60.5	70.6	14.3	2.5
4	9	C	-	-	-	120	78	1.14	8.00	9.14	12.5	1.5
		PROLONGED CHEMO STIMULATION (10-20mins)										
5	15	CHEMO	30	52	7.25	103	92	1.89	5.89	7.77	24.3	2.0
6	23	CHEMO	35	49	7.22	103	92	1.09	6.80	7.89	13.8	2.5
7	26	C	-	-	-	-	-	1.68	6.34	8.00	20.7	1.6
8	28	C	-	-	-	-	-	1.09	2.91	4.00	27.1	2.8
9	33	C	98	38	7.31	-	-	2.17	8.17	10.3	21.0	2.8
10	40	C	-	-	-	120	60	3.54	21.0	24.6	14.4	4.0
11	42	BARO	85	35	7.23	82	114	9.14	36.6	45.8	20.0	4.5
12	46	C	-	-	-	-	-	2.86	12.7	15.5	18.4	3.0
	50	CYCLOHEXIMIDE (50mg/kg)										
13	55	C	-	-	-	-	-	1.94	11.7	13.7	14.2	2.5
14	61	C	-	-	-	120	78	0.97	8.29	9.26	10.5	2.5
15	63	BARO	60	44	7.14	82	142	8.23	34.4	42.6	19.3	5.0
16	69	C	-	-	-	120	67	0.74	4.34	5.09	14.6	2.0
		PROLONGED CHEMO STIMULATION (70-80mins)										
17	75	CHEMO	26	58	7.14	103	107	4.51	20.0	24.5	18.4	2.5
18	79	CHEMO	22	53	7.12	103	107	4.46	23.4	27.8	16.0	2.2

DC7 12kg (M)

19	83	C	-	-	-	-	-	0.80	6.86	7.66	10.4	1.0
20	89	C	-	-	-	-	-	0.80	3.03	3.83	20.9	1.2
21	91	C	-	-	-	120	64	0.00	3.37	3.37	0.0	1.0
22	93	BARO	69	46	7.17	86	114	5.37	14.3	19.7	27.2	1.5
23	97	C	-	-	-	-	-	1.03	1.83	2.86	36.0	0.8
	101	ACTH (iv) (0.9 I.U./kg)										
24	106	C	-	-	-	-	-	2.00	5.60	7.60	23.3	2.0
25	111	C	-	-	-	-	-	2.29	5.37	7.66	29.9	2.0
26	113½	BARO	69	44	7.58	120	57	9.54	27.8	37.4	25.5	2.0
27	120	C	-	-	-	82	103	11.7	4.23	15.9	73.4	1.0

DC8 6kg (F)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	129	-	5.37	9.26	14.6	36.7	1.5
2	1½	BARO	190	39	7.33	79	-	8.69	26.9	35.5	24.4	1.5
3	5	C	-	-	-	129	-	5.94	7.77	13.7	43.3	0.9
4	6½	BARO	144	39	7.34	79	-	15.8	41.0	56.9	27.9	1.3
5	10	C	-	-	-	129	-	3.09	8.00	11.1	27.8	1.0
6	11½	BARO	-	-	-	79	-	13.6	41.3	54.9	24.8	1.4
7	14	C	144	37	7.39	129	-	6.86	2.06	8.91	76.9	0.9
		PROLONGED CHEMO STIMULATION (15-25mins)										
8	20	CHEMO	37	48	7.28	129	-	217.4	456.4	673.8	32.5	2.4
9	24	CHEMO	22	53	7.15	129	-	107.0	238.4	345.4	31.0	1.1
10	31½	C	-	-	-	129	-	8.80	44.3	53.1	16.6	
11	33	BARO	-	-	-	71	-	12.5	36.0	48.5	25.7	1.4
12	35	C	-	-	-	125	-	4.91	22.2	27.1	18.1	1.5
13	36½	BARO	144	42	7.30	71	-	13.4	52.3	65.7	20.4	2.0
14	43	C	-	-	-	129	-	2.51	12.1	14.6	17.2	1.4
15	44	BARO	-	-	-	64	-	20.9	64.2	85.1	24.6	1.4
16	50	C	-	-	-	125	-	1.37	12.7	14.1	9.8	0.7
17	51½	BARO	166	37	7.33	68	-	10.9	44.2	55.1	19.7	1.5
	53	ATROPINE (20µg/kg)										
18	55	C	-	-	-	129	-	2.74	11.7	14.4	19.0	-
19	56½	BARO	-	-	-	68	-	30.4	87.2	117.6	25.9	1.5

	DC8	6kg (F)										
20	60	C	-	-	-	125	-	3.77	16.2	20.0	18.9	0.8
21	61½	BARO	-	-	-	64	-	35.7	116.3	152.0	23.5	1.4
	63	HEXAMETHONIUM (2mg/kg)										
22	65	C	-	-	-	-	-	1.71	7.66	9.37	18.3	1.2
23	70	C	-	-	-	129	-	2.17	7.31	9.49	22.9	1.3
24	71½	BARO	-	-	-	64	-	5.60	23.2	28.8	19.4	1.4

CAT1 3kg (F)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	149	49					
2	$2\frac{1}{2}$	BARO	435	28	7.44	85	148					
3	10	C	440	34	7.36	154	78	TOO LOW FOR RATIOS				
4	$12\frac{1}{2}$	CHEMO	114	68	7.05	154	151					
5	20	C	-	-	-	149	49					
6	$22\frac{1}{2}$	BARO	425	28	7.34	76	163	3.77	0.11	3.89	97.1	-
7	32	C	385	32	7.34	134	68	0.00	0.00	0.00	-	-
8	$34\frac{1}{2}$	CHEMO	117	116	6.89	134	176	4.91	6.40	11.3	43.4	-
9	43	C	-	-	-	144	59	9.37	9.94	19.3	48.5	-
10	$45\frac{1}{2}$	BARO	370	27	7.32	76	81	19.8	9.94	29.7	66.5	-
11	53	C	480	36	7.31	110	68	0.00	0.91	0.91	0.0	-
12	$55\frac{1}{2}$	CHEMO	0	120	6.96	110	167	32.3	62.7	95.1	34.0	-

CAT2 2.9kg (F)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	-	-	-	-	-	10.4	4.67	15.0	68.9	0.5
2	2½	BARO	135	35	7.31	-	-	3.17	3.32	6.49	48.8	0.5
3	10	C	-	-	-	146	42	10.5	0.87	11.3	92.4	0.6
4	12½	BARO	152	25	7.35	69	145	5.47	4.10	9.57	57.1	1.2
5	20	C	150	29	7.46	145	45	4.90	7.09	12.0	40.9	0.6
6	22½	CHEMO	5	65	6.90	145	45	5.79	5.42	11.2	51.7	0.6
7	30	C	-	-	-	144	36	1.26	2.95	4.22	30.0	0.5
8	32½	BARO	150	33	7.35	69	170	3.91	3.44	7.35	53.2	1.4
9	40	C	-	-	-	144	36	3.12	3.27	6.39	48.8	0.5
10	42½	CHEMO	5	65	6.90	144	70	3.02	4.51	7.53	40.1	0.5
11	50	C	-	-	-	145	50	3.07	3.89	6.96	44.1	0.5
12	52½	BARO	108	23	7.29	69	150	5.03	4.27	9.30	54.1	1.4
13	58	C	-	-	-	-	-	-	-	-	-	
14	60	S.Ch	119	26	7.32	145	100	5.60	30.3	35.9	15.6	0.4
15	70	C	115	32	7.21	145	43	10.2	17.5	27.7	36.8	0.6
16	72½	S.Ch	-	-	-	145	200	19.6	37.1	56.6	34.5	0.2
17	75	C	-	-	-	145	-	14.0	26.4	40.4	34.7	0.2

S.Ch = Suberyl Choline (100µg/min into carotid perfusion)

CAT3 3.4kg (F)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	335	21	7.50	181	65	0.85	0.85	1.71	49.7	1.4
2	$2\frac{1}{2}$	BARO	225	23	7.44	86	129	2.62	1.09	3.71	70.6	2.4
3	10	C	215	25	7.45	171	38	0.79	0.50	1.29	62.0	0.6
4	$12\frac{1}{2}$	CHEMO	46	41	7.14	171	106	1.00	1.76	2.74	36.0	1.2

CAT4 2.9kg (F)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	157	25	7.47	152	85	2.17	3.72	5.89	36.7	-
2	2½	BARO	155	23	7.51	112	167	3.42	5.00	8.42	40.7	-
3	10	C	142	26	7.44	152	105	1.22	0.77	1.99	61.4	-
4	12½	BARO	135	28	7.42	88	136	4.24	5.10	9.34	45.4	-
5	30	C	137	23	7.44	152	69	3.76	4.00	7.76	48.4	-
6	32½	CHEMO	0	50	6.94	152	115	5.83	9.62	15.5	37.8	-
7	40	C	107	26	7.39	148	100	2.83	6.45	9.28	30.5	-
8	42½	BARO	133	21	7.42	92	121	4.97	6.55	11.5	43.1	-
9	45	C	126	26	7.33	152	74	4.24	5.34	9.58	44.4	-
10	49	CHEMO	0	67	6.85	152	162	3.10	5.31	8.41	36.8	-
	50	HEXAMETHONIUM (2mg/kg)										
11	60	C	139	30	7.38	152	69	1.26	2.39	3.65	34.5	-
12	62½	BARO	-	-	-	80	74	1.32	1.95	3.27	40.3	-
13	70	C	137	30	7.36	152	79	1.13	1.44	2.57	44.0	-
14	72½	CHEMO	0	68	6.95	152	110	1.55	2.65	4.11	37.6	-
	75	HEXAMETHONIUM (4mg/kg)										
15	80	C	116	32	7.28	156	100	0.60	1.58	2.18	27.7	-
16	82½	BARO	126	32	7.30	84	105	0.52	1.21	1.73	30.2	-
17	90	C	144	29	7.34	156	90	0.56	1.47	2.03	27.5	-
18	92½	CHEMO	0	78	6.87	156	131	1.13	3.55	4.68	24.3	-
		ATROPINE (100µg/kg)										
		DEATH										

CAT5 3.5kg (M)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	310	21	7.53	152	65	0.46	2.76	3.22	14.3	1.2
2	2½	BARO	-	-	-	119	197	5.43	6.47	11.9	45.6	2.8
3	13	C	385	24	7.54	152	58	1.66	2.44	4.08	40.4	0.8
4	15½	CHEMO	8	64	6.68	152	135	1.24	6.15	7.40	16.8	1.8
5	23	C	180	27	7.36	144	65	7.10	4.30	11.4	62.3	1.4
6	25½	BARO	-	-	-	81	81	6.65	3.95	10.6	62.8	1.6
7	33	C	-	-	-	156	61	1.43	2.33	3.76	38.1	1.2
8	35½	CHEMO	42	50	7.07	156	74	1.43	2.33	3.76	38.1	1.2
9	46	C	160	35	7.27	152	55	1.49	2.09	3.58	41.6	1.0
10	48½	BARO	-	-	-	78	123	6.47	4.80	11.3	57.4	2.2
11	53	C	-	-	-	167	39	4.81	4.19	9.00	53.4	0.8
12	55½	CHEMO	51	48	7.15	167	55	3.97	3.28	7.25	54.8	0.8
	64	HEXAMETHONIUM (2mg/kg)										
13	69	C	160	35	7.27	152	45	0.14	0.95	1.09	12.5	0.4
14	71½	BARO	-	-	-	81	52	0.15	1.33	1.49	10.0	0.5
15	77	C	-	-	-	152	55	0.25	1.14	1.39	17.9	0.6
16	79½	CHEMO	6	58	6.86	152	90	4.68	13.3	18.0	26.1	0.8
		ATROPINE (20µg/kg)										
		DEATH										

CAT6 3.2kg (M)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	230	23	7.52	146	109	1.46	1.41	2.87	50.9	-
2	2½	BARO	-	-	-	76	152	2.33	1.44	3.77	61.7	-
3	10	C	-	-	-	143	98	6.34	1.12	7.46	85.0	-
4	12½	CHEMO	5	48	7.09	143	148	0.00	0.77	0.77	0.0	-
5	20	C	130	31	7.38	141	85	5.51	0.54	6.05	91.0	-
6	22½	BARO	-	-	-	76	146	0.00	0.54	0.54	0.0	-
7	31	C	225	30	7.27	139	98	3.15	0.44	3.59	87.8	-
8	33½	CHEMO	37	62	6.97	139	172	3.15	0.44	3.59	87.8	-
	35	ATROPINE (50ug/kg)										
9	47	C	120	34	7.23	146	57	2.98	1.56	4.54	65.7	-
10	49½	BARO	-	-	-	78	191	11.4	3.99	15.4	74.1	-
11	55	C	132	33	7.35	146	83	2.23	2.61	4.84	46.0	-
12	57½	CHEMO	46	52	7.08	146	189	6.73	5.82	12.5	53.6	-
	60	ATROPINE (1mg/kg)										
13	64	C	145	29	7.32	150	89	1.82	1.07	2.89	63.1	-
14	66½	BARO	-	-	-	78	193	9.97	3.28	13.3	75.2	-
15	74	C	145	33	7.34	148	74	1.15	2.53	3.68	31.2	-
16	76½	CHEMO	49	48	7.12	113	148	3.79	4.52	8.31	45.6	-
	78	HEXAMETHONIUM (2mg/kg)										
17	79	C	127	32	7.30	137	46	1.23	0.65	1.88	65.6	-
18	81½	BARO	-	-	-	78	89	1.84	0.82	2.66	69.3	-
19	100	C	150	30	7.32	150	50	1.97	2.49	4.46	44.2	-
20	102½	CHEMO	39	58	6.95	150	50	2.75	4.14	6.89	39.9	-

	CAT6	3.2kg (M)										
21	109	C	-	-	-	-	-	3.53	6.93	10.5	33.7	-
22	114½	C	-	-	-	-	-	1.15	0.11	1.26	91.5	-
	120	HEXAMETHONIUM (4mg/kg)										
23	127	C	125	30	7.36	150	54	1.04	0.65	1.69	61.5	-
24	129½	BARO	-	-	-	78	98	0.05	2.96	3.01	1.6	-
25	137	C	133	29	7.36	124	59	1.78	0.00	1.78	100.0	-
26	139½	CHEMO	5	64	6.86	124	74	1.50	1.57	3.07	48.7	-
27	149	C	-	-	-	-	-	1.88	0.00	1.88	100.0	-
28	161	C	150	31	7.26	-	-	2.31	0.00	2.31	100.0	-
29	166	C	-	-	-	-	-	1.61	0.00	1.61	100.0	-
30	174	C	-	-	-	-	-	1.40	0.00	1.40	100.0	-
	176	ACTH (5I.U.)										
31	179	C	165	27	7.38	-	-	1.01	0.00	1.01	100:0	-
32	185	C	-	-	-	-	-	1.14	0.00	1.14	100.0	-
33	190	C	165	30	7.35	-	-	1.78	0.00	1.78	100.0	-
34	195	C	-	-	-	-	-	5.28	1.01	6.28	84.0	-
35	206	C	-	-	-	-	-	4.45	2.98	7.43	59.9	-
36	219	C	-	-	-	150	113	4.01	0.29	4.30	93.3	-
37	221½	BARO	-	-	-	76	183	2.66	8.20	10.9	24.5	-

CAT6 3.2kg (M)

38	231	C	-	-	-	148	107	2.23	0.00	2.23	100.0	-
39	233½	CHEMO	5	65	6.86	148	163	2.97	3.21	6.18	48.1	-
	235	ATROPINE (40ug/kg)										
40	245	C	-	-	-	120	76	-	-	-	-	-
		PROLONGED CHEMO STIMULATION (250-260mins)										
41	250	CHEMO	5	65	6.86	120	111	5.04	1.65	6.68	75.4	-
42	258	CHEMO	5	65	6.86	120	111	5.50	1.16	6.66	82.6	-
	265	HEXAMETHONIUM (6mg/kg)										
43	270	C	-	-	-	120	48	1.72	0.00	1.72	100.0	-
44	272½	CHEMO	5	65	6.86	120	59	2.88	1.62	4.50	64.0	-
45	279	CHEMO	-	-	-	-	-	5.59	14.9	20.5	27.3	-
46	281½	CHEMO	-	-	-	-	-	6.26	12.6	18.9	33.2	-

CAT7 3.0kg (F)

No.	TIME (mins)	TEST	CAROTID ARTERIAL BLOOD $P O_2$	$P CO_2$	pH	CSP	SBP	ADRENAL OUTPUT (ng/kg.min) NA	A	TOTAL	NA %	FLOW (mls)
1	0	C	180	34	7.06	-	-	0.00	1.53	1.53	0.0	-
2	2½	BARO	-	-	-	-	-	2.27	4.90	7.17	31.5	-
3	12	C	-	-	-	-	-	0.55	1.18	1.72	31.3	-
4	14½	CHEMO	58	52	6.89	-	-	0.59	1.76	2.35	25.1	-
5	22	C	150	32	7.20	-	-	0.74	1.96	2.70	27.8	-
6	24½	BARO	-	-	-	-	-	2.27	2.98	5.25	43.2	-
7	32	C	160	30	7.21	-	-	0.78	1.41	2.19	35.5	-
8	34½	CHEMO	8	73	6.75	-	-	0.55	2.51	3.06	18.3	-
	40	ATROPINE (100µg/kg)										
9	46	C	140	30	7.11	-	-	1.06	1.49	2.55	41.4	-
10	48½	BARO	-	-	-	-	-	2.19	1.18	3.37	65.6	-
11	56	C	285	33	7.17	-	-	1.02	1.49	2.51	39.9	-
12	58½	CHEMO	10	78	6.55	-	-	0.82	0.98	1.80	45.7	-
	65	ATROPINE (1mg/kg)				-	-					
13	73	C	375	24	7.21	-	-	1.02	3.13	4.15	24.3	-
14	75½	BARO	-	-	-	-	-	1.21	3.17	4.39	27.7	-
15	83	C	495	28	7.08	-	-	0.71	1.72	2.43	28.8	-
16	85½	CHEMO	11	74	6.50	-	-	0.67	4.19	4.86	13.3	-
	90	HEXAMETHONIUM (6mg/kg)										
17	94	C	106	14	7.22	-	-	7.4	42.7	50.2	14.7	-
18	96½	CHEMO	7	30	6.84	-	-	13.0	40.0	53.0	24.5	-
19	99	AFTER CHEMO	-	-	-	-	-	34.8	129.0	163.8	21.3	-

BARO Releases
Before any drug

EXPT	TEST	P O_2	P CO_2	pH	↓CSP	↑SBP	↑NA	↑A	↑TOTAL	% NA	FLOW X↑
DR1	2	103	32	7.42	57	107	0.84	6.98	7.82	10.7	1.14
	6	126	31	7.38	65	67	-	-	-	-	1.00
DR2	2	122	37	7.37	-	-	0.00	1.39	1.39	0.0	1.00
	6	83	41	7.35	-	-	2.64	6.58	9.21	28.6	1.17
DR3	2	90	33	7.40	61	110	-	-	-	-	1.00
	6	89	34	7.41	67	104	3.28	27.3	30.6	10.7	1.22
DR4	2	89	60	7.21	57	102	2.11	4.99	7.19	29.7	2.73
	8	84	52	7.26	57	72	1.69	6.93	8.62	19.6	1.92
	14	94	48	7.30	57	62	0.78	2.72	3.50	22.4	1.76
	20	95	54	7.24	-	-	5.88	22.6	28.5	20.6	1.86
DR5	2	100	43	7.34	80	66	0.20	7.11	7.31	2.8	1.44
	4	-	-	-	75	53	0.41	8.48	8.89	4.6	1.33
	6	110	24	7.36	75	53	0.25	6.45	6.70	3.8	1.62
	7	119	24	7.46	-	-	-	-	-	-	1.64
	10	111	23	7.37	80	72	0.61	3.40	4.01	15.2	1.94
	12	105	38	7.32	70	97	0.10	5.99	6.10	1.7	1.89

BARO Releases
Before any drug

EXPT	TEST	P O_2	P CO_2	pH	↓CSP	↑SBP	↑ NA	↑A	↑TOTAL	% NA	FLOW ↓↑
DA1	2	395	53	7.28	58	78	1.88	2.79	4.35	40.3	1.09
	4	420	54	7.24	37	47	1.30	4.07	5.36	24.0	1.10
	6	410	51	7.24	80	69	2.01	4.64	6.66	30.2	1.10
	8	430	58	7.23	65	41	0.54	2.89	3.28	16.7	1.23
	10	390	44	7.32	65	62	-	-	-	-	1.20
DA2	2	220	45	7.31	69	100	7.65	19.2	26.8	28.5	1.45
	4	422	40	7.35	74	107	-	-	-	-	1.31
	6	385	42	7.35	79	113	6.55	20.4	26.9	24.3	2.13
DA3	2	365	46	7.26	39	95	18.9	72.6	91.6	20.6	1.63

BARO Releases
Before any drug

EXPT	TEST	P O_2	P CO_2	pH	↓CSP	↑SBP	↑NA	↑A	↑TOTAL	% NA	FLOW X ↑
DC1	2	365	37	7.38	66	166	0.29	5.19	5.48	5.4	1.50
	4	70	39	7.39	62	143	0.54	9.40	9.94	5.4	1.72
DC2	3	-	-	-	-	-	18.2	117.6	135.8	13.4	1.40
DC3	3	81	35	7.31	68	81	-	-	54.1	38.8	2.25
DC4	3	74	38	7.30	43	52	9.00	53.2	62.1	14.5	1.00
DC5	3	396	46	7.29	53	100	7.85	18.9	26.8	29.3	1.93
DC6	3	-	-	-	47	33	3.60	9.53	13.1	27.4	3.59
DC7	3	135	37	7.25	45	75	9.47	54.9	64.4	14.7	1.79
DC8	2	190	39	7.33	50	-	3.31	17.6	20.9	15.8	1.00
	4	144	39	7.34	50	-	9.94	33.3	43.2	23.0	1.44
	6	-	-	-	50	-	10.5	33.3	43.8	24.0	1.40

BARO Releases
After Guanethidine

EXPT	TEST	P O_2	P CO_2	pH	↓CSP	↑SBP	↑NA	↑A	↑TOTAL	% NA	FLOW X↑
DR1	10	154	36	7.40	61	0	1.59	5.36	6.94	22.8	1.00
	14	140	35	7.41	66	26	0.86	3.98	4.84	17.7	1.00
DR2	10	155	42	7.32	-	-	6.13	10.9	17.0	36.0	1.05
DR3	10	78	39	7.39	44	0	8.29	20.1	28.4	29.2	1.00
	14	82	39	7.39	61	0	5.99	20.4	26.4	22.7	1.24
	18	96	35	7.43	61	27	4.44	21.1	25.5	17.4	1.18

BARO Releases After ACTH

EXPT	TEST	P O_2	P CO_2	pH	↓CSP	↑SBP	↑NA	↑A	↑TOTAL	% NA	FLOW X↑
DA1	12	365	41	7.30	71	53	0.00	9.10	9.10	0.0	1.16
	14	400	45	7.30	68	59	6.67	5.90	12.6	53.3	1.17
	16	375	40	7.31	65	60	4.36	9.80	14.1	30.7	1.10
	18	430	42	7.28	65	41	4.39	4.50	8.90	49.5	1.09
	20	455	47	7.29	68	28	0.00	20.2	20.2	0.0	1.13
	22	455	47	7.27	22	12	5.13	7.30	12.4	41.5	1.05
	24	425	40	7.29	74	28	4.14	15.2	19.4	21.3	1.15
DA2	8	390	44	7.31	79	88	4.82	41.3	46.1	10.5	1.92
	10	395	43	7.34	79	58	7.94	33.8	41.7	19.0	1.27
	12	360	42	7.28	79	61	6.88	28.3	35.1	19.6	2.41
	14	365	44	7.29	79	43	16.0	51.3	67.3	23.8	1.50
	16	360	39	7.31	79	64	15.2	53.3	68.5	22.2	1.69
	18	365	38	7.31	79	61	6.04	39.2	45.2	13.3	1.64
	20	365	38	7.31	79	61	10.5	21.0	31.6	33.3	1.52
DA3	8	-	-	-	39	62	11.0	94.5	105.5	10.4	2.35
	10	335	42	7.35	39	23	12.8	71.0	83.8	15.3	2.00

BARO Releases After Prolonged Chemo Stimulation

EXPT	TEST	$P O_2$	$P CO_2$	pH	↓CSP	↑SBP	↑NA	↑A	↑TOTAL	% NA	FLOW X ↑
DC3	9	85	38	7.32	68	96	-	-	77.8	40.5	2.00
DC4	11	86	31	7.31	43	34	19.9	107.0	128.0	15.5	3.50
DC5	10	109	51	7.23	53	89	7.39	39.7	47.1	15.7	1.63
DC6	9	110	43	7.23	47	55	3.08	3.55	6.63	46.5	2.50
DC7	11	85	35	7.23	38	54	0.56	1.56	2.12	5.6	1.13
DC8	11	-	-	-	58	-	3.70	0.00	3.75	-	1.00
	13	144	42	7.30	54	-	8.49	30.1	38.6	22.1	1.33
	15	-	-	-	65	-	13.4	52;3	65.7	20.4	1.43
	17	166	37	7.33	57	-	9.49	31.5	41.0	23.1	1.67

After Cycloheximide

DC2	11	119	23	7.41	-	-	38.0	122.9	160.8	23.6	3.25
DC4	15	82	32	7.27	43	29	5.98	11.4	17.3	34.5	1.00
DC5	16	126	52	7.20	64	75	7.89	35.0	42.9	18.4	2.58
DC6	15	70	52	7.18	47	38	1.22	2.38	3.60	33.9	1.83
DC7	15	60	44	7.14	38	64	7.26	26.1	33.3	21.8	2.00

				After Second Chemo Stimulation (+ Cycloheximide)							
DC2	16	105	38	7.32	-	-	10.3	91.6	101.9	10.1	4.00
DC4	21	96	41	7.24	43	26	18.5	65.3	83.8	22.1	2.67
DC5	22	127	56	7.15	57	98	5.19	18.2	13.0	39.9	2.93
DC6	21	125	45	7.31	47	26	0.12	0.87	1.00	11.8	2.67
DC7	22	69	46	7.17	34	50	5.37	10.9	16.4	32.7	1.50
				After ACTH (+ Cycloheximide)							
DC5	28	123	62	7.12	61	96	0.00	26.7	26.7	0.0	2.39
DC7	26	69	44	7.58	38	46	7.25	22.4	29.7	24.4	1.00
				After Atropine							
DC8	19	-	-	-	61	-	27.7	75.5	103.2	26.8	-
	21	-	-	-	61	-	31.9	100.1	132.0	24.2	1.75

CHEMO Releases Before any Drug (1 min Duration)

EXPT	TEST	$P O_2$	$P CO_2$	pH	↓ CSP	↑ SBP	↑ NA	↑A	↑TOTAL	% NA	FLOW ↑↑
DR1	4	28	37	7.40	0	33	-	-	-	-	1.00
	8	27	30	7.37	0	67	0.60	1.48	2.08	28.9	1.10
DR2	4	32	58	7.34	-	-	1.05	1.31	2.36	44.5	1.11
	8	33	58	7.28	-	-	0.13	1.33	1.46	8.8	1.28
DR3	4	41	43	7.37	0	0	0.57	2.24	2.80	20.2	1.00
	8	34	44	7.40	0	0	0.27	0.09	0.36	75.0	1.07
DR4	5	42	70	7.18	0	58	0.98	3.79	4.77	20.5	1.52
	11	22	68	7.10	0	80	3.45	6.51	9.96	34.6	1.70
	17	41	76	7.05	0	57	3.01	23.1	26.1	11.5	2.35
	23	2	104	6.98	-	-	5.51	23.4	28.9	19.0	1.20

CHEMO Releases After Guanethidine (1 min Duration)

DR1	12	27	43	7.35	0	6	-	-	-	-	1.10
	16	24	40	7.38	0	27	0.66	2.49	3.15	21.1	1.09
DR2	12	27	62	7.26	-	-	4.37	10.8	15.2	28.7	1.08
DR3	12	35	41	7.38	0	0	1.91	3.13	5.04	37.9	1.00
	16	33	41	7.39	0	0	0.00	6.29	6.29	0.0	1.00
	20	36	40	7.42	0	0	0.36	1.70	2.06	17.5	1.00

Concurrent Releases During Prolonged CHEMO Stimulation

EXPT	TEST	$P O_2$	$P CO_2$	pH	↓CSP	↑SBP	↑NA	↑A	↑TOTAL	% NA	FLOW X↑
DC3	5	35	52	7.24	-	-	-	-	2.48	24.2	1.00
	6	35	52	7.24	-	-	-	-	2.84	42.3	1.67
DC4	5	47	44	7.22	0	30	1.92	0.00	1.92	-	1.00
	6	43	44	7.22	0	30	0.22	0.00	0.22	-	1.00
	17*	37	44	7.16	0	-22	4.44	20.0	24.4	18.2	1.26
	18*	37	44	7.16	0	-22	0.27	9.65	9.93	2.8	1.00
DC5	5	55	74	7.26	35	43	6.46	46.0	52.5	12.3	1.52
	6	43	88	7.30	35	43	4.80	43.0	47.8	10.0	1.19
	18*	31	76	7.20	35	11	4.11	20.5	24.6	16.7	1.25
	19*	31	76	7.20	35	11	2.74	22.6	25.4	10.8	1.25
DC6	5	44	47	7.27	0	26	1.30	4.15	5.43	23.9	1.48
	6	40	49	7.28	0	26	0.00	7.00	7.00	0.0	1.00
	17*	37	58	7.14	0	4	0.17	0.17	0.34	50.0	1.60
	18*	28	62	7.18	0	4	0.17	0.00	0.17	-	1.00
DC7	5	30	52	7.25	17	14	0.75	0.00	0.75	-	1.33
	6	35	49	7.22	17	14	0.00	0.00	0.00	-	1.67
	17*	26	58	7.14	-	-	3.77	15.7	19.4	19.4	1.25
	18*	22	53	7.12	-	-	3.72	19.1	22.7	16.5	1.10
DC8	8	37	48	7.28	0	-	210.5	454.4	664.9	31.7	2.67
	9	22	53	7.15	0	-	100.1	236.3	336.4	29.8	1.67

(* CHEMO Stimulation after introduction of Cycloheximide)

SLANCHNIC NERVE STIMULATION RELEASES

EXPT	TEST	ARTERIAL BLOOD $P O_2$	ARTERIAL BLOOD $P CO_2$	ARTERIAL BLOOD pH	↓CSP	↑SBP	ADRENAL OUTPUT(ng/kg.min) ↑NA	↑A	↑TOTAL	%NA	FLOW X↑
DN1	2	425	44	7.37	-	-	123.8	420.4	544.3	22.8	-
	4	-	-	-	-	-	61.6	265.3	327.0	18.9	-
	6	-	-	-	-	-	51.2	162.6	213.8	21.3	-
	8	410	42	7.25	-	-	51.2	160.4	211.6	24.2	-
	10	425	46	7.31	-	-	48.4	154.3	202.7	23.9	-
	12	-	-	-	-	-	66.9	165.6	232.5	28.8	-
DN2	2	420	38	7.31	-	-	0.97	9.20	10.2	9.6	-
	4	-	-	-	-	-	7.43	37.7	45.1	16.5	-
	7	-	-	-	-	-	18.3	98.0	116.3	15.7	-
	9	-	-	-	-	-	20.8	84.8	105.6	19.7	-
	11	415	39	7.37	-	-	1.94	4.46	6.40	30.4	-
	13	420	38	7.37	-	-	1.43	1.54	2.97	48.1	-
After Hexamethonium (2mg/kg)											
DN2	15	-	-	-	-	-	1.09	2.46	3.54	30.6	-
After Atropine (2 g/kg)											
DN1	14	427	43	7.30	-	-	36.4	127.7	164.1	22.2	-
After Atropine and Hexamethonium											
DN1	16	-	-	-	-	-	0.0	0.0	0.0	-	-
DN2	17	-	-	-	-	-	0.0	0.0	0.0	-	-

BARO Releases Before any Drug

EXPT	TEST	P O_2	P CO_2	pH	↓CSP	↑SBP	↑NA	↑A	↑TOTAL	% NA	FLOW X↑
CAT1	6	425	28	7.34	73	114	3.77	0.11	3.89	97.1	-
	10	370	27	7.32	68	22	10.4	0.00	10.4	100.0	-
CAT2	2	135	35	7.31	-	-	-	-	-	-	1.00
	4	152	25	7.35	77	103	-	-	-	-	2.00
	8	150	33	7.35	75	134	2.65	0.49	3.13	84.7	2.80
	12	108	23	7.29	76	100	1.96	0.38	2.34	83.8	2.80
CAT3	2	225	23	7.44	95	64	1.76	0.24	2.00	88.2	1.71
CAT4	2	155	23	7.51	40	82	1.25	1.28	2.53	49.5	-
	4	136	28	7.42	64	31	3.02	5.33	8.35	36.2	-
	8	133	21	7.42	56	21	2.14	0.10	2.24	95.5	-
CAT5	2	310	21	7.53	33	132	4.97	3.71	8.68	57.4	1.83
	6	180	27	7.36	63	16	5.22	1.62	6.84	76.6	1.14
	10	160	35	7.27	74	68	4.98	2.71	7.62	64.7	2.20
CAT6	2	230	23	7.52	70	43	0.87	0.03	0.90	95.5	-
	6	130	31	7.38	65	61	-	-	-	-	-
CAT7	2	180	34	7.06	-	-	2.27	3.37	5.64	40.4	-
	6	150	32	7.20	-	-	1.53	1.02	2.55	59.7	-

BARO Releases

After Hexamethonium

EXPT	TEST	P O_2	P CO_2	pH	↓CSP	↑SBP	↑NA	↑A	↑TOTAL	% NA	FLOW ×↑
CAT4	12	139	30	7.38	72	5	0.04	0.00	0.04	-	-
	16	126	32	7.30	72	5	-	-	-	-	-
CAT5	14	160	35	7.27	71	7	0.01	0.38	0.39	2.6	1.25
CAT6	18	127	32	7.30	35	43	0.61	0.17	0.78	78.2	-
	24	125	30	7.36	72	44	0.00	2.31	2.31	0.0	-

After Atropine

CAT6	10	120	34	7.23	68	134	8.42	2.43	10.9	77.5	-
	14	145	29	7.32	72	104	8.15	2.21	10.4	78.6	-
CAT7	10	140	30	7.11	-	-	1.13	0.00	1.13	100.0	-
	14	375	24	7.21	-	-	0.19	0.04	0.23	82.6	-

CHEMO Releases
Before any Drug

EXPT	TEST	P O_2	P CO_2	pH	↓CSP	↑SBP	↑ NA	↑ A	↑TOTAL	% NA	FLOW X↑
CAT1	8	117	116	6.89	0	108	4.91	6.40	11.3	43.4	-
	12	0	120	6.96	0	99	32.3	61.8	94.2	34.3	-
CAT2	6	5	65	6.90	0	0	-	-	-	-	1.00
	10	5	65	6.90	0	34	0.00	1.24	1.24	0.0	1.00
CAT3	4	46	41	7.14	0	68	0.21	1.26	1.44	14.3	2.00
CAT4	6	0	50	6.94	0	46	2.07	5.62	8.74	23.7	-
CAT5	4	8	64	6.68	0	77	0.00	3.71	3.71	0.0	2.25
	8	42	50	7.07	0	13	-	-	-	-	1.00
	12	51	48	7.15	0	16	-	-	-	-	1.00
CAT6	4	5	48	7.09	0	50	-	-	-	-	-
	8	37	62	6.97	0	74	-	-	-	-	-
CAT7	4	58	52	6.89	-	-	0.04	0.58	0.63	5.9	-
	8	8	73	6.75	-	-	0.00	1.10	1.10	0.0	-

SCh Releases
Before any Drug

CAT2	14	119	26	7.32	0	50	2.53	26.4	28.9	8.7	
	16	119	32	7.21	0	157	9.40	19.6	28.9	32.5	

CHEMO Releases
After Hexamethonium

EXPT	TEST	P O_2	P CO_2	pH	↓CSP	↑SBP	↑ NA	↑ A	↑ TOTAL	% NA	FLOW X ↑
CAT4	14	0	68	6.95	0	31	0.42	1.12	1.64	27.3	-
	18	0	78	6.87	0	41	0.57	2.08	2.65	22.0	-
CAT5	16	6	58	6.86	0	35	4.43	12.2	16.6	26.7	1.33
CAT6	20	39	58	6.95	0	0	0.78	1.65	2.43	32.1	-
	26	5	64	6.86	0	15	0.00	1.57	1.57	0.0	-
	44	5	65	6.86	0	11	1.16	2.17	3.33	34.9	-
	45	5	65	6.86	0	11	3.87	15.4	19.3	27.3	-
	46	5	65	6.86	0	11	4.54	13.1	17.7	25.6	-
CAT7	18	7	30	6.84	-	-	12.3	38.3	50.6	24.3	-

After Atropine

CAT6	12	46	52	7.08	0	106	4.50	3.21	7.71	58.4	-
	14	49	48	7.12	35	74	2.64	1.99	4.63	57.0	-
	41	5	65	6.86	0	35	2.81	2.09	4.89	57.3	-
	42	5	65	6.86	0	35	3.27	1.60	4.85	67.3	-
CAT7	16	11	74	6.50	-	-	0.00	2.47	2.47	0.0	-
	12	10	78	6.55	-	-		no release			

ISOLATED DOG ADRENAL GLANDS
(hpp TMA)

	TEST No	DOSE (Molar)	CONTROL OUTPUT TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)	INCREMENTAL RELEASE TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)
WEST (JUNE'75)										
A2	7	10^{-7}M	257	14	36	221	191	24	46	145
	9	10^{-6}M	222	16	36	186	5015	27	1354	3661
A3	2	10^{-7}M		no release						
	5	5 X 10^{-7}M	540	15	81	459	933	28	261	672
	8	10^{-6}M	493	12	59	434	1431	27	386	1045
A5	4	10^{-7}M	887	22	195	692	87	28	24	63
	8	5 X 10^{-7}M	888	19	169	719	4349	21	913	3436
A12	4	5 X 10^{-7}M	102	31	32	70	117	25	29	88
	5	10^{-6}M	207	20	41	166	436	12	53	383
WEST & WAITE (AUGUST'74)										
			78	29	23	55	84	35	29	55
			165	42	69	96	102	33	34	68
		DOSES	1062	25	266	796	232	38	88	144
		10^{-8}-10^{-6}M	268	16	43	225	253	9	23	230
			283	35	99	184	294	12	35	259
			54	10	5	49	1752	25	438	1314

ISOLATED DOG ADRENAL GLANDS
(hpp TMA-cont)

TIBENHAM (SPRING'74)

DOSES (Molar)	CONTROL OUTPUT TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)	INCREMENTAL RELEASE TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)
	31	15	5	26	640	22	141	499
	33	22	7	26	698	32	223	475
	43	20	9	34	751	33	248	503
	228	13	30	198	858	32	275	583
	284	31	88	196	2064	27	557	1507
10^{-7}-10^{-5}M	258	14	36	222	2710	15	407	2303
	376	12	45	331	3890	24	934	2956
	308	23	71	237	4290	24	1030	3260
	236	20	47	189	4823	30	1447	3376
	522	21	110	412	6882	34	2340	4542
	157	11	17	140	7645	17	1300	6345

ISOLATED DOG ADRENAL GLANDS (METHACHOLINE)

	TEST No	DOSE (Molar)	CONTROL OUTPUT TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)	INCREMENTAL RELEASE TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)
WEST (JUNE/JULY'75)										
A2	8	10^{-6}M	262	19	50	212	1828	22	402	1426
A3	3	10^{-6}M	900	15	135	775	599	32	192	407
	6	2 X 10^{-6}M	649	24	156	493	621	33	205	416
	9	5 X 10^{-6}M	617	15	93	524	1473	38	560	913
A3	5	5 X 10^{-7}M	955	24	229	726	200	24	48	152
	2	10^{-6}M	1314	19	250	1064	348	21	73	275
	9	2 X 10^{-6}M	1119	25	280	839	660	22	145	515
A8	4	10^{-6}M	114	28	32	82	83	20	17	66
	5	2 X 10^{-6}M	146	22	32	114	110	11	12	98
WEST & WAITE (AUGUST'74)										
		10^{-7}M	61	15	9	52	164	24	39	125
		10^{-7}M	94	23	22	72	342	27	92	250
		10^{-6}M	802	20	160	642	692	32	221	471

ISOLATED DOG ADRENAL GLANDS
(METHACHOLINE-cont)

TIBENHAM (SPRING'74)

DOSES (Molar)	CONTROL OUTPUT TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)	INCREMENTAL RELEASE TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min
	247	25	62	185	93	9	8	85
	725	23	167	558	127	25	32	95
	373	22	82	291	176	34	60	116
10^{-6}-10^{-8}M	100	20	20	80	181	19	34	147
	327	27	88	239	239	32	76	163
	159	27	43	116	241	15	36	205
	97	11	11	86	639	21	134	505
	161	18	30	131	647	31	301	446
	592	23	136	456	1002	23	230	772

ISOLATED CAT ADRENAL GLANDS
(hpp TMA)

	TEST No	DOSE (Molar)	CONTROL OUTPUT TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)	INCREMENTAL RELEASE TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)
C1	1	10^{-6}M	175	39	68	107	145	75	109	36
	7	10^{-6}M	87	39	34	53	132	71	94	38
	3	2 X 10^{-6}M	125	36	45	80	260	62	161	99
	5	2 X 10^{-6}M	97	32	31	66	191	69	132	59
C2	1	2 X 10^{-6}M	270	37	100	170	1085	65	705	380
	3	2 X 10^{-6}M	88	47	41	47	1036	67	694	342
C3	5	2 X 10^{-7}M		no release						
	7	5 X 10^{-7}M	21	42	9	12	34	40	14	20
	2	10^{-6}M	10	33	3	7	170	38	65	105
	9	10^{-6}M	9	37	3	6	85	73	62	23
	3	2 X 10^{-6}M	22	43	10	12	541	63	341	200
	14	5 X 10^{-6}M	10	60	6	4	143	58	83	60
	12	10^{-5}M	30	30	9	20	2255	73	1646	609
C4*	4	2 X 10^{-6}M	115	25	29	86	372	42	156	206
	6	2 X 10^{-6}M	115	25	29	86	351	42	147	204
	8	5 X 10^{-6}M	79	27	21	58	303	40	121	182
	10	5 X 10^{-6}M	75	39	29	46	297	45	134	163
	14	10^{-5}M	37	53	20	17	327	51	167	160
		HEXAMETHONIUM (3 X 10^{-4}M)								
	16	5 X 10^{-6}M		no release						
	19	10^{-5}M		no release						

ISOLATED CAT ADRENAL GLANDS
(hpp TMA-cont)

	TEST No	DOSE (Molar)	CONTROL OUTPUT TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)	INCREMENTAL RELEASE TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)
C6*	2	5×10^{-6}M	110	34	37	73	100	41	41	59
	5	10^{-5}M	119	36	43	76	168	59	99	69
	7	10^{-5}M	97	31	30	67	192	60	115	77
		HEXAMETHONIUM (3×10^{-4}M)								
	10	10^{-5}M		no release						
C7	2	10^{-6}M	168	29	49	119	397	44	175	222
	9	10^{-6}M	32	31	10	22	174	41	71	103
	4	2×10^{-6}M	105	28	29	76	516	52	268	248
	6	2×10^{-6}M	73	53	39	34	499	47	235	264
	12	5×10^{-6}M	37	38	14	23	711	64	455	256
	13	5×10^{-6}M	58	37	21	37	386	84	324	62
	15	10^{-5}M	21	70	15	6	734	60	440	294
		ATROPINE (3×10^{-5}M)								
	17	5×10^{-6}M	20	80	16	4	48	44	21	27
	20	5×10^{-6}M	22	77	17	5	39	45	18	21

* Killed with Nembutal

ISOLATED CAT ADRENAL GLANDS
(METHACHOLINE)

	TEST No	DOSE (Molar)	CONTROL OUTPUT TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)	INCREMENTAL RELEASE TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)
C1	2	10^{-5}M	159	20	32	127	215	47	101	114
	6	2 X 10^{-5}M	85	33	28	57	295	45	133	162
	4	10^{-4}M	115	40	46	69	648	40	259	389
C2	2	2 X 10^{-5}M	122	28	34	88	312	55	172	140
	4	2 X 10^{-5}M	41	57	23	18	325	29	94	231
C3	6	2 X 10^{-6}M		no release						
	4	5 X 10^{-6}M	15	52	8	7	134	29	39	95
	10	5 X 10^{-6}M	24	53	13	11	101	25	25	76
	8	10^{-5}M	13	55	7	6	227	29	66	161
	1	2 X 10^{-5}M	35	45	16	19	368	18	66	302
	13	5 X 10^{-5}M	12	57	7	5	59	38	22	36
	11	10^{-4}M	29	18	5	24	285	33	94	191
C4*	1	10^{-5}M	138	25	35	103	170	32	54	116
	2	10^{-5}M	133	25	33	100	313	35	110	203
	13	10^{-5}M	68	35	24	44	69	30	21	48
	3	2 X 10^{-5}M	111	24	27	84	286	31	89	197
	5	2 X 10^{-5}M	121	25	30	91	283	29	82	201
	11	5 X 10^{-5}M	70	39	27	43	494	29	143	351

* Killed with Nembutal

ISOLATED CAT ADRENAL GLANDS
(METHACHOLINE-Cont)

	TEST No	DOSE (Molar)	CONTROL OUTPUT TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)	INCREMENTAL RELEASE TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)
C4 (Cont)		HEXAMETHONIUM (3×10^{-4}M)								
	15	5×10^{-5}M	57	26	15	42	162	38	62	100
	18	10^{-4}M	35	39	14	21	170	35	60	110
C6*	1	5×10^{-5}M	135	27	36	99	163	36	59	104
	4	10^{-4}M	105	34	36	69	334	35	117	217
	6	10^{-4}M	113	21	24	89	429	36	154	275
		HEXAMETHONIUM (3×10^{-4}M)								
	9	10^{-4}M	129	24	31	98	236	37	87	199
	11	10^{-4}M	88	42	37	51	156	34	53	103
	13	10^{-4}M	88	45	40	44	145	41	59	86
C7	9	5×10^{-6}M	34	23	8	26	172	44	76	96
	1	10^{-5}M	32	62	20	12	482	32	154	328
	7	10^{-5}M	41	35	14	27	259	37	96	163
	3	2×10^{-5}M	114	35	29	85	457	35	160	297
	5	2×10^{-5}M	272	24	65	207	322	28	90	232
		ATROPINE (3×10^{-5}M)								
	16	2×10^{-5}M		no release						
	19	5×10^{-5}M		no release						

ISOLATED CAT ADRENAL GLANDS
CARBOCHOL

	TEST No	DOSE (Molar)	CONTROL OUTPUT TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)	INCREMENTAL RELEASE TOTAL CA (ng/min)	% NA	NA (ng/min)	A (ng/min)
C4*	7	10^{-5}M	81	44	35	46	200	36	72	128
	9	10^{-5}M	70	39	27	43	190	42	80	110
	12	10^{-4}M	290	21	61	229	1009	53	535	474
		HEXAMETHONIUM (3×10^{-4}M)								
	17	2×10^{-5}M	34	43	15	19	89	39	35	54
	20	2×10^{-5}M	21	49	10	11	67	40	27	40
	22	5×10^{-5}M	31	41	13	18	138	49	68	70
C6*	3	2×10^{-5}M	120	30	36	84	296	53	157	139
	8	2×10^{-5}M	74	40	30	44	395	43	170	125
		HEXAMETHONIUM (3×10^{-4}M)								
	11	2×10^{-5}M	103	36	37	66	121	34	41	80
	12	2×10^{-5}M	99	47	47	52	101	35	35	66
	14	2×10^{-5}M	85	60	51	34	77	28	22	45
C7	10	5×10^{-6}M	44	41	18	26	393	59	232	161
	13	5×10^{-5}M	72	52	37	35	184	62	114	70
	6	10^{-5}M	26	53	14	12	401	45	180	221
		ATROPINE (3×10^{-5}M)								
	18	10^{-5}M		no release						
	21	4×10^{-5}M	29	64	19	10	30	8	2	28

* Killed with Nembutal

DOG
ISOLATED GLANDS

HYDROCORTISONE -

TEST No.	DOSE (µg/ml)	CONTROL OUTPUT TOTAL CA (ng/min)	% NA IN CONTROL	PEAK INCREMENT (ng/min)	AVERAGE INCREMENT (ng/min)	% NA IN RELEASE
GLAND A2						
3	30 µg/ml	339	14	57	41	16
4	30	293	15	110	83	47
1	50	530	16	253	173	12
2	50	327	16	236	206	36
5	100	310	21	567	424	28
6	100	293	21	383	259	29
10	150	388	26	537	371	34
GLAND A3						
14	25 µg/ml	334	19	111	90	27
4	30	780	15	139	92	25
1	50	1604	19	297	269	8
12	50	379	20	397	256	5
11	75	431	15	321	222	5
7	100	516	16	613	548	19
13	100	359	19	1158	787	15
10	150	505	13	1905	1548	11
GLAND A4						
1	25 µg/ml		no release			
2	50	707	27	656	553	26
3	100	473	21	756	684	23
GLAND A5						
6	25 µg/ml	930	24	129	85	34
3	50	1055	22	504	459	8
12	50	520	20	115	77	25
10	75	723	24	246	203	27
7	100	976	20	1305	1194	12
11	100	580	21	638	596	12

DOG
ISOLATED GLANDS

HYDROCORTISONE

GLAND A6

1	50µg/ml	613	20	134	87	48
2	100	370	24	529	469	22
3	150	495	22	478	428	23

GLAND A7

3	30µg/ml		no release			
1	50	498	16	309	282	13
4	75	402	11	441	369	12
2	100	471	14	1238	1061	13
5	150	381	22	1838	1461	10
6	200	439	14	1887	1723	18

GLAND A8

3	30µg/ml	152	23	79	56	15
1	50	204	21	270	197	16
2	100	174	24	413	325	16

GLAND A9

3	30µg/ml		no release			
1	50	258	22	84	60	38
2	100	270	28	198	126	3
4	100	176	22	160	99.6	14
5	150	172	25	403	269	6

GLAND A10

1	100µg/ml	175	21	219	174	18
2	150	150	38	272	237	28

DOG
ISOLATED GLANDS
HYDROCORTISONE

GLAND A11

1	100µg/ml	284	25	445	374	15
2	150	437	11	1528	1112	12
3	200	490	14	1475	1179	11

GLAND A12

1	100µg/ml	113	22	244	207	19
2	150	158	24	489	397	15
3	200	102	31	657	491	20

DOG
ISOLATED GLANDS

ALDOSTERONE
(Compared with HYDROCORTISONE)

TEST No.	DOSE (µg/ml)		CONTROL OUTPUT TOTAL CA (ng/min)	% NA IN CONTROL	PEAK INCREMENT (ng/min)	AVERAGE INCREMENT (ng/min)	% NA IN RELEASE
GLAND A9							
3	30	HC		no release			
1	50	HC	258	22	85	60	38
2	100	HC	270	28	198	126	3
4	100	HC	176	22	160	100	14
5	150	HC	172	25	403	269	6
6	5	Aldosterone		no release			
7	6	Aldosterone		no release			
8	10	Aldosterone		no release			
GLAND A10							
5	50	HC		no release			
3	100	HC	175	21	219	174	18
6	150	HC	150	38	272	237	28
1	5	Aldosterone		no release			
2	7.5	Aldosterone		no release			
4	10	Aldosterone		no release			
GLAND A11							
3	50	HC		no release			
1	100	HC	284	25	445	374	15
2	150	HC	437	11	1528	1112	12
5	200	HC	490	14	1475	1179	11
4	7.5	Aldosterone		no release			
6	10	Aldosterone		no release			
7	20	Aldosterone		no release			

DOG
ISOLATED GLANDS

DEOXYCORTICOSTERONE

TEST No.	DOSE (µg/ml)	CONTROL OUTPUT TOTAL CA (ng/min)	% NA IN CONTROL	PEAK INCREMENT (ng/min)	AVERAGE INCREMENT (ng/min)	% NA IN RELEASE
AUGUST 1974. 3 GLANDS						
	40µg/ml	54	27	193	157	37
	40	169	13	120	91	38
	50	216	35	177	135	35
	50	134	22	79	37	30
	100	287	27	708	479	37
	100	220	16	163	112	32

APPENDIX VII.

MANUFACTURERS

Instruments

1) Anaesthetic infusion pumps - Palmer Injection Infusion Pump Apparatus,
C.F. Palmer, Lane End Road, High Wycombe,
Buckinghamshire, England.

2) Watson-Marlow pump - W.M. MHRE 200, Falmouth, Cornwall.

3) Blood Pressure Transducer - Physiological Pressure Measurement System,
Electronic Instrumentation Group,
Bell and Howell, Ltd.,
Basingstoke, England.

4) Ultraviolet Recorder - Visicorder 2206 - AC
Honeywell

5) Servo - control system -

a) Operational amplifiers - Ancon Ltd.,
Cheltenham, England.

b) Power supply - Fenlow Electronics Ltd.,
Weybridge, Surrey.

6) Blood Micro System - Acid - Base Analyser, PHM 71
Radiometer, Copenhagen, Denmark.

7) Aminco - Bowman Spectrophotofluorometer - American Instrument Company Inc.,
Silver Spring,
Maryland,
U.S.A.

8) Chemical Processing Unit - Unicam AC 60,
Pye Unicam Ltd.,
Cambridge, England.

9) "Evapomix" - Evapo - Mix,
Buckler Instruments,
Fort Lee, New Jersey, U.S.A.

10) "Whirlymix" - Rotamixer,
Hook and Tucker, Ltd., U.K.

Reagents - suppliers

1) B.D.H. Chemicals Ltd, Poole, Dorset.

2) Koch - Light Laboratories Ltd., Colnbrook, Buckinghamshire, England.

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PUBLICATIONS

Catecholamine release from the Adrenal Medulla

Selective Release

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Microscopy 33 (i) (January - June 1976)

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GLOSSARY

CATECHOLAMINES - refers only to noradrenaline and adrenaline* (see p 17)

BARORECEPTORS - refers to the carotid sinus stretch receptors. (see p 15)

BARORECEPTOR TEST - reduction of perfusion pressure in the carotid bifurcation resulting in withdrawal of stretch receptor inhibitory tone. (see p 92)

CHEMORECEPTORS - refers to the arterial chemoreceptors of the carotid body. (see p 10)

CHEMORECEPTOR TEST - perfusion of the carotid bifurcations with hypoxic blood resulting in stimulation of the arterial chemoreceptors. (see p 92)

SODIUM DITHIONITE - reducing agent used for lowering the oxygen tension of the blood perfusing the carotid bifurcations. (see p 109)

THI / Trihydroxyindole reaction - used for producing the characteristic fluorophors in our catecholamine assay. (see p 121)

BAL / British anti-lewisite - dimercaptopropanol - reagent we used to stabilise the fluorophors in the THI reaction. (see p 142)

METHACHOLINE - Acetyl B Methyl Choline - muscarinic agonist drug. (see p 152)

hppTMA - meta-hydroxyphenylpropyl trimethylammonium - nicotinic agonist drug. (see p 152)

SYNACTHEN - synthetic analogue of corticotrophin / adrenocorticotrophic hormone, ACTH. (see p 103)

CYCLOHEXIMIDE - inhibitor of protein synthesis which rapidly blocks the secretion of corticosteroids from the adrenal cortex. (see p 104)

Whole Animal Experiments D = Dog

DR 1-5 - alternate baroreceptor and chemoreceptor tests.

DN 1&2 - serial periods of electrical stimulation of the splanchnic nerve to the adrenal gland.

DN 3 - effect of Synacthen administration on serial periods of splanchnic nerve stimulation.

DA 1-3 - effect of Synacthen administration on serial baroreceptor tests.

DC 1 - effect of prolonged chemoreceptor stimulation on serial baroreceptor tests.

DC 2-7 - effect of cycloheximide on the response to prolonged chemoreceptor stimulation.

DC 8 - effect of prolonged chemoreceptor stimulation on serial baroreceptor tests with the other gland denervated.

CAT 1-8 - alternate baroreceptor and chemoreceptor tests.

*The other catecholamines such as dopamine are only found in small amounts in the mammalian adrenal gland. However, it has recently been appreciated that dopamine reaches concentrations an order greater than does noradrenaline in peripheral plasma but this would not be detected by the THI assay. (see "The Adrenal Gland", Handbook of Physiology series, 1975).